EXHIBIT 20 B

In re Application of: McDaniel, et al.

(Transmittal of Appeal Brief - page 1 of 3)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial f Filed: For:	No.:	08/252,384 June 1, 1994 Recombinant Organo Anhydrase and Meth		Group No.: Examiner:	1814 C. Low		
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٦	TRANSMIT	TAL OF APPEAL BRI	EF (PATENT A	PPLICATIO	N-37 CFR 192)		
1. Notice	Transmitted herewith in triplicate is the APPEAL BRIEF in this application with respect to the of Appeal filed on November 25, 1994.						
NOTE:	application, or p	shell, within 2 months from the patent under reexemination, or lee a brief in <u>triplicate</u> . 37 CFR	within the time allows	d for response to	1.191 in an application, reissu the action appealed from, if suc		
2.	STATUS (OF APPLICANT					
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3.	FEE FOR F	FILING APPEAL BRIE	F				
		37 CFR 1.17(f), the fee		eal Brief is:			
		ll entity or than a small entity	\$140.00 \$280.00				
	·		Appeal	Brief fee due	\$ 140.00		
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4.	EXTENSION OF TERM						
NOTE:	The tin 1.191	The time periods set forth in 37 CFR 1.192(a) are subject to the provision of § 1.136 for patent applications. 37 CFI 1.191(d). Also see Notice of November 5, 1985 (1060 O.G. 27).					
		(complete (a) or (b) as app	licable)				
(a)	X	Applicant petitions for an extension of time under 37 CFR 1.136 (fees: 37 CFF 1.17(a)-(d) for the total number of months checked below:					
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		OR					
(b)		Applicant believes that no extension of time is required. However, this conditional petition is being made to provide for the possibility that Applicant has inadvertently overlooked the need for a petition for extension of time.					
5 .	TOT	AL FEE DUE					
	The total fee due is: Appeal brief fee \$ 140.00 Extension fee (if any) \$ 435.00		TOTAL FEE DUE:	\$ 575.00			
6. FEE PAYMENT							
	\(\times\)	Attached is a check in the sum of \$575.00. Charge Account No. 03-2769 the sum of \$_					
		A duplicate of this transmittal is attached	_				

7. FEE DEFICIENCY

NOTE: If there is a fee deficiency and there is no authorization to charge an account, additional fees are necessary to cover the additional time consumed in making up the original deficiency. If the maximum, six-month period has expired before the deficiency is noted and corrected, the application is held abandoned. In those instances where authorization to charge is included, processing delays are encountered in returning the papers to the PTO Finance Branch in order to apply these charges prior to action on the cases. Authorization to charge the deposit account for any fee deficiency should be checked. See the Notice of April 7, 1986, (1065 O.G. 31-33).

6. If any additional extension and/or fee is required, this is a request therefor and to charge Account No. 03-2769.

AND/OR

If any additional fee for claims is required, charge Account No. 03-2769.

SIGNATURE OF ATTORNEY

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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

APPLICANT:

McDaniel, et al.

SERIAL NO.:

08/252,384

FILING DATE:

June 1, 1994

CLIENT:

TxTox,Inc.

FOR:

Recombinant Organophosphorus Acid Anhydrase and Methods of Use

The date stamp of the mail room of the U.S. Patent & Trademark Office hereon will acknowledge receipt of APPEAL BRIEF AND EXHIBITS, TRANSMITTAL OF APPEAL BRIEF (PATENT APPLICATION - 37 CFR 192) and CHECK IN THE AMOUNT OF \$575.00, sent by U.S. first-class mail, postage prepaid, on April 25, 1995.



MRS. WILLETTE L. NORMAN (CSM) CONLEY, ROSE & TAYON, P.C. P. O. BOX 3267 HOUSTON, TEXAS 77253-3267

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BRIEF (PATENT APPLICATION - 37 CFR 192) and CHECK IN THE AMOUNT OF acknowledge receipt of APPEAL BRIEF AND EXHIBITS, TRANSMITTAL OF APPEAL \$575.00, sent by U.S. first-class mail, postage prepaid, on April 25, 1995.

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APPLICATION FOR UNITED STATES LETTERS PATENT

FOR

RECOMBINANT ORGANOPHOSPHORUS ACID ANHYDRASE AND METHODS OF USE

Ву

Inventors: C. Steven McDaniel Frank M. Raushel and James R. Wild

Assignee: TEXAS A&M UNIVERSITY SYSTEM

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The U.S. Army Research Office provided funding used in part for this invention under contract 21288-LS (6/84-6/86) and contract DAALO3-87-K-0017 (12/86-12/89). Accordingly, the Federal Government may have certain rights in this invention pursuant to 35 U.S.C. 202.

BACKGROUND OF THE INVENTION

A. FIELD OF THE INVENTION

This invention relates to genetic engineering. The invention relates particularly to a cloned bacterial gene and heterologous expression of the gene in diverse biological hosts using a variety of expression vectors. The invention relates further to uses of the recombinant enzyme encoded by the bacterial gene. More particularly, the invention relates to the use of the recombinant gene and/or enzyme to detoxify and detect organophosphorus neurotoxins or to detect microorganisms capable of detoxifying organophosphorus compounds.

B. <u>DESCRIPTION OF RELATED ART</u>

Synthetic organophosphorus neurotoxins are used extensively as agricultural and domestic pesticides. Organophosphorus compounds are also common components of nerve gases found in chemical warfare arsenals.

A variety of problems have arisen due to the use of organophosphorus compounds. Chief among these problems is the lack of an effective means for safe and thorough disposal. Currently, organophosphorus compounds are detoxified by either basic hydrolysis, dilution in aqueous solutions or incineration. These techniques are not efficient and may cause environmental pollution. Massive

stockpiles of aging nerve gas containers are particularly difficult to detoxify using current methodology.

Another problem arises from the difficulty in protecting personnel, supplies and equipment from potentially hazardous organophosphorus vapors. This is a serious problem under field conditions experienced by military personnel under threat of chemical attack. One means for providing such protection used currently is by enzymatic detoxification using the enzyme DFPase of giant squid axon. However, the availability of DFPase is limited making such protection very expensive.

A similar problem arises when organophosphorus compounds are used to treat crops near beehives. The unprotected insects are susceptible to oversprays which may seriously curtail honey production. Similarly, other beneficial insects such as silk worms can be seriously endangered by use of organophosphorus compounds near silk manufacturing operations or on food plants used in silk manufacture. No adequate means exists for protecting such insect-based operations.

Another set of problems regarding organophosphorus compounds arises out of the necessity of detecting such compounds in a variety of settings. Not the least of these problems involves the detection of potentially hazardous organophosphorus vapors. Means currently in use to detect organophosphorus compounds typically require bulky, sophisticated equipment. The use of such equipment, particularly in field settings, is impractical. Even when such equipment can be used, such as in a laboratory setting, means for rapidly confirming the presence of organophosphorus compounds in trace amounts are required.

Awareness of a unique set of problems associated with the biolabile organophosphorus pesticides has recently arisen. The effectiveness of organophosphorus pesticide applications is compromised by the presence of soil bacteria capable of rapidly detoxifying these pesticides. No means are currently available to detect such bacteria prior to pesticide application. Without a means of testing for bacteria capable of detoxifying organophosphorus pesticides, applications of organophosphorus compounds to crops may be ineffective.

In an effort to resolve some of these problems, naturally occurring bacterial isolates capable of metabolizing the organophosphorus compounds have received considerable attention (1,2) since they provide the possibility of both environmental and in situ detoxification (reviewed in 3). [As used herein, numbers in parentheses refer to references in the bibliography unless indicated otherwise.] Pseudomonas and Flavobacterium species have been shown to possess the ability to degrade an extremely broad spectrum of organophosphorus phosphotriesters as well as thiol esters. However, none of these strains have shown the ability to use neurotoxins as sole nutrient sources. Consequently, selection of strains suitable for recombinant DNA research has been difficult.

Two bacterial strains from the closely related genera Pseudomonas and Flavobacterium have been found to encode (opd) genes on large plasmids (4,5,6). The genetic locations of the degradative genes are unknown in other bacteria (7,8). The isolation and subcloning of opd from these large, degradative plasmids (40 to 65 kilobases in size) has proven to be very difficult (5,6,9). In particular, expression of the opd gene in heterologous backgrounds has not been feasible on a commercial basis.

There have been numerous attempts to characterize the enzyme encoded by the <u>opd</u> gene using crude extracts of the native bacteria (10,11). However, limitations arise using this approach due to two factors. First, these soil bacteria are difficult to culture. Second reliance on crude extracts for the necessary characterization of the enzyme can be misleading and costly. With the increasing use of organophosphorus neurotoxins in modern society, means for detoxifying and detecting such compounds and means for maximizing their effectiveness are needed.

SUMMARY OF THE INVENTION

The present invention provides an organophosphorus detoxifying (opd) gene of the DNA sequence set forth in Figure 1; a recombinant bacterial organophosphorus acid anhydrase (OPA) enzyme derived from the opd gene; a collection of expression vectors comprising the opd gene; a collection of transformed cells comprising the opd gene on an expression vector; and, transgenic organisms comprising the opd gene on an expression vector. Furthermore, the invention provides several methods for using the gene, vectors, cells and organisms of the invention, namely: a method for making commercial quantities of OPA; a method for purifying the OPA; methods for using either recombinant opd microorganisms or the purified OPA to detoxify organophosphorus compounds; a method for detecting opd-containing microorganisms; a method for detecting organophosphorus compounds in residue analysis or in air samples; a method for protecting beneficial insects using either recombinant microorganisms or recombinant protein or the opd gene itself; and, a method for co-formulating organophosphorus pesticides prior to application.

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows the nucleotide sequence of the opd
gene.

Figure 2 illustrates the activity of certain opd subclones. Sequences are adjusted to align vector DNA on either side of the opd subclone. Deletions are indicated by open space between brackets. The putative coding region for the opd gene is indicated by shading, and the sense direction is shown by arrows.

Figure 3 shows the construction of the <u>opd</u> expression vector for use in insect cells.

Figure 4 shows the mortality curve for infected and uninfected caterpillars.

Figure 5(a) shows the master plate of <u>Pseudomonas</u> <u>diminuta</u>. The arrow indicates a colony subsequently shown to lack OPA activity; Figure 5(b) is a filter lift of the master plate in Figure 5(a) which has been allowed to develop 4-nitrophenol coloration and is subsequently photographed using UV-illumination. The arrow indicates the same colony as described in Figure 5(a); Figure 5(c) is an image produced by overlapping (eclipsing) Figures 5(a) and 5(b). The round, bright colony in the lower right-hand corner of the image corresponds to that described in Figures 5(a) and 5(b).

Figure 6 shows the derivation of plasmids containing opd from Pseudomonas diminuta or Flavobacterium sp. where (a) is the P. diminuta plasmid pCMS1, and (b) is the Flavobacterium sp. plasmid.

Figure 7 shows a Southern blotting and hybridization of the 1.3-kb probe with plasmid DNAs from P. diminuta and a Flavobacterium sp.

Figure 8 shows a Southern blotting and hybridization of the 0.9-kb probe with plasmid DNAs from P. diminuta and a Flavobacterium sp.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a biological means of detoxifying and detecting organophosphorus compounds. The invention provides as well a means for protecting susceptible organisms from organophosphorus poisoning. The invention also provides means for detecting organophosphorus-detoxifying microorganisms and for coformulating pesticides for maximal efficiency. In order to achieve these ends, the invention relies on the heterologous expression of the specific DNA sequence containing the organophosphorus detoxifying (opd) gene encoding the recombinant organophosphorus acid anhydrase enzyme (OPA).

Heterologous Expression of the opd Gene

The opd gene is isolated by first isolating the native plasmid DNA of an organophosphorus-detoxifying bacterium such as <u>Pseudomonas diminuta</u> or <u>Flavobacterium</u> sp. (ATCC 27551). Surprisingly, the inventors discovered that, in at least two cases, these native plasmids carry the identical <u>opd</u> gene (This is further elaborated in Harper, et al. 1988. <u>Appl</u>. <u>Environ</u>. <u>Microbiol</u>. <u>54</u>:2586-2589). The two DNA sequences are invariant but the native plasmid vectors are different. It is this precise DNA fragment which was isolated by the inventors and which allowed heterologous expression.

The opd gene fragment derived from the native plasmid DNA above is purified by any one of a number of methods known to those of skill in the art and is then inserted into an expression vector chosen for its ability to transform a given cell. Typically, the initial subcloning would be made by transforming the bacterial cell <u>E. coli</u>. The use of a <u>PstI</u>-derived fragment substantially free of extraneous DNA is critical to the success of the initial cloning since the heterologous expression of a <u>Pseudomonas</u> or <u>Flavobacterium</u> gene in <u>E. coli</u> is difficult to achieve. This discovery allowed the inventors to succeed in obtaining heterologous expression where others had failed to do so. (This is explained in McDaniel, et al. 1988. <u>J. Bacteriol</u>. 170:2306-2311).

In order to carry out the steps necessary to adequately express the <u>opd</u> gene, it is necessary to determine the sequence of the DNA encoding it. This can be achieved by methods known to those skilled in the art and is illustrated in Figure 1 for the <u>PstI</u> fragment containing the <u>opd</u> gene.

It is equally important to determine the correct reading frame prior to manipulating the gene in order to increase expression. Determining the correct reading frame can be accomplished by isolating the gene's protein product and amino acid sequencing the protein using techniques known to those skilled in the art. Preferably one isolates a fusion polypeptide the <u>opd</u> portion of which when sequenced confirms the proper reading frame. (This is detailed in McDaniel, et al. 1988. <u>J. Bacteriol</u>. <u>170</u>:2306-2311).

Armed with the DNA sequence and the known reading frame, a number of vector systems with different types of plasmids and different types of promoters are constructed

and placed into \underline{E} . \underline{coli} . It is very difficult to improve on the expression that is observed in the normal soil bacteria source. This is evident if one compares the various bacterial strains and vectors in Table I. Extensive manipulation of these constructs demonstrates that the protein is deposited in the bacterial host membranes and that the \underline{E} . \underline{coli} membrane limits the amount of protein produced.

Table 1. Expression of <u>opd</u> in heterologous biological systems.

Biological host ^a	Expression	Promoter	Activity
P. diminuta MG (25°C)	pCMS1 (native)	opdP ^d	2.08
<u>P. diminuta</u> MĢ (25°C)	cured strain	none	<0.001
E. coli JM103 (37°C)	M13mp10 (phage)	<u>lacP</u>	0.013
E. coli JM103 (37°C)	no phage	none	<0.001
E. coli MC4100 (37°C)	pLH540 (plasmid)	<u>tacP</u>	0.020
E. coli MC4100 (25°C)	pLH540 (plasmid)	<u>tacP</u>	1.10
E. coli MC4100 (25°C)	no plasmid	none	<0.01
Sf9 cell culture (25°C)	pLH1170 ^e	hedP	12.50
Sf9 cell culture (25°C)	(uninfected) ^f	none	<0.01

Temperature of growth conditions indicated in parentheses b Promoter utilized to express opd cistron
Enzymatic activity is express as 1 µmole of paraoxon converted to p-nitrophenol per milligram of protein per minute (units/mg) where $E_{400} = 17,000 M^{-1} cm^{-1}$.
dNative pseudomonad plasmid encoding opd
eBaculoviral transfection of plasmid construction
fTested cells alone from uninfected and 360B-gal transfected cells

Thus, it is necessary to transfer the <u>opd</u> gene into an alternate host such as the baculoviral vector system. A recombinant DNA molecule is constructed using the polyhedron gene promoter to control synthesis of the <u>opd</u> transcript and this is transformed into insect tissue culture cells in the presence of a native helper virus. The transformed cultures produce high levels of OPA, up to 10-fold better than the original bacterial source (see Table I above).

Insect tissue cultures are screened for the presence of a clone demonstrating opd activity. Since paraoxon is degraded to form p-nitrophenol (yellow) plus diethyl thiophosphate, the relative rates of OP-hydrolysis by each clone is screened in microculture (250 microliters). Thus, cultured cell lines that are capable of producing 50 to 100 times the activity of the recombinant bacterial cultures may be selected.

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Use of Expression Vectors Comprising the opd Gene

The expression vectors comprising an <u>opd</u> gene fragment may be used to produce transgenic eukaryotic organisms. Such transgenic organisms are those that have

had the genetic material of another organism inserted into their cells in some manner and have propogated the foreign DNA by incorporating it into their own cellular DNA. Typically, the foreign DNA is incorporated into the transgenic organism using a movable genetic element such as a transposon or a virus which is capable of infecting the transgenic host. For example, a transgenic fruit fly is produced by injecting an expression vector comprising a transposon carrying the opd gene into the fruit fly embryo cells. In another example, an expression vector comprising the baculovirus transfer vector carrying the opd gene is injected into Fall Army worm caterpillars. The transgenic insect is preferably a beneficial insect such as the honey bee or silk worm.

The recombinant OPA enzyme is produced using specific expression vectors. Each such vector must be comprised of a promoter and a start codon recognized by the host cell. The promoter may be selected from lac, tac, amp, the heat shock promoter of a P-element of Drosophila, or the baculovirus polyhedron gene promoter. In addition, the expression vector should include an opd DNA fragment in the correct orientation and reading frame with respect to the promoter sequence to allow translation of the opd gene. In one example, the expression vector comprises a plasmid such as pBR322. In another example, the expression vector comprises a bacteriophage M13.

In another example where transfer of the <u>opd</u> gene is to be effected in <u>Drosophila</u>, a plasmid carrying a transposon which in turn carries the <u>opd</u> gene sequence is required. Preferably, the <u>Drosophila</u> vector is a Pelement with a heat shock promoter controlling <u>opd</u>. In the Fall Army worm, the expression vector is derived from the baculovirus enabling its use in transforming the

insect cell lines. The baculovirus vector may be one of any of several baculovirus transfer vectors.

Characterization of the Recombinant OPA and its Use

With the increased levels of production provided by the recombinant gene heterologously expressed in an insect host, purification of the enzyme in large amounts may be achieved. For example, from 8g of insect cells infected with the baculovirus/opd vector, 2.7 mg of homogeneous enzyme may be obtained with an overall yield of 75% after 1500-fold purification.

The invention provides for purified enzyme with a specific activity of 3200 units/mg. Kinetic constants $(k_{cat}, k_m, k_{cat}/k_m)$ can be calculated describing the catalytic efficiency of the enzyme. The kinetic values associated with the purified enzyme (specific activity = 3200 units/mg) assuming a molecular weight of approximately 39,000 can be calculated as:

$$K_{cat} = 2100 \text{ sec}^{-1}$$

$$K_{cat}/K_{m} = 4 \times 10^{7} M^{-1} sec^{-1}$$

The pure enzyme is then used in a series of studies known to those skilled in the art to determine the mechanism of action and substrate specificity. (This is described more fully in Lewis, V.E., et al. 1988. <u>Biochemistry 27</u>: 1591-1597 and in Example III below).

This type of information allows one to predict what can and can not be accomplished with the enzyme relative to various types of substrates. For example, the purified enzyme may be used to determine whether this enzyme degrades mammalian neurotoxins such as Soman or VX.

An evaluation of the kinetic parameters for diisopropyl phosphonofluoridate (DFP) hydrolysis is also accomplished with the recombinant organophosphorus anhydrase using either a fluoride ion electrode or $^{19}{\rm F-NMR}$. The K $_{\rm m}$ for DFP hydrolysis at pH 7.0 is 0.12 \pm 0.02 mM and the V $_{\rm max}$ is 3.6% of the maximal rate of hydrolysis of paraoxon. The K $_{\rm m}$ for paraoxon hydrolysis is 0.012 \pm 0.001 mM under the same reaction conditions for the enzyme produced in $\underline{\rm E.~coli}$.

Other Uses of the Invention

According to the method of this invention, detoxification using the purified OPA enzyme or the opd-containing microorganisms can be attained for a wide range of organophosphorus compounds. Detoxification is achieved by the initial hydrolysis across the susceptible bond of the organophosphorus compound. For example, detoxification of parathion may be achieved by conversion of parathion to p-nitrophenol and diethyl thiophosphoric acid. Detoxification using the purified OPA enzyme has the advantage of avoiding the potential release of genetically engineered microorganisms into the environment.

In one application of the invention, either the purified enzyme or the <a href="https://open.com/open.

microorganism reacts with the organophosphorus contaminant thereby detoxifying it. Likewise, in another application of the invention, the matrix-bound OPA or opd recombinant-microorganism is incorporated in a gas mask device to protect personnel such as pesticide applicators or soldiers exposed to hazardous levels of organophosphorus compounds present as vapors. In another application, a matrix-bound OPA or opd recombinant microorganism may be used to filter potentially contaminated air entering a closed environment such as a building or vehicle. In still another application, matrix-bound OPA or opd-containing recombinant microorganism contained within a solid phase column is used to detoxify an effluent. Such an effluent is a waste water stream from a locality where organophosphorus compounds are being manufactured, applied, or destroyed.

The invention may also be used to decontaminate a variety of loci by disseminating either the purified OPA or the opd-containing microorganism onto the locus. Any manner of dissemination may be used, but preferably the enzyme or the opd-containing recombinant bacteria will be sprayed preferably in an inert solution to better facilitate the spray. The potentially contaminated locus can range from a generally contaminated soil or body of water to military or commercial pesticide-application equipment. The locus can equally well be a pre- or post-harvest crop, an animal (including a human) or clothing. The locus may even be a stored foodstuff which has been treated or otherwise contaminated with an organophosphorus pesticide.

In another application of the invention, the purified enzyme or the recombinant opd microorganism may be a concentrated liquid form or a solid form such as a solid tablet. In such a concentrated formulation, the invention

may be used to detoxify containers such as those used in commercial, agricultural, or domestic pesticide applications. Such a concentrated formulation may also be used in detoxifying containers of spent nerve gases. Concentrated formulations of the purified OPA enzyme or opd-containing recombinant bacteria may also be used as antidotes for poisons. These concentrated formulations may be used after poisoning or as a pretreatment for animals or humans prior to exposure to sub-acute or lethal doses of organophosphorus compounds.

In still another application of the invention, the recombinant microorganism comprising both the opd gene and an antibiotic resistance marker on the same vector is used in a plate assay to detect bacterial colonies capable of detoxifying organophosphorus compounds. Preferably, the control opd-microorganism will be mixed with samples of indigenous bacteria to provide an internal control to samples randomly obtained from soil, water, feeds, etc. By plating out aliquots of samples spiked with the control opd bacteria on both non-selective (no antibiotic) and selective (containing antibiotic) media, it is possible to calculate precisely the number of control opd-containing bacteria and to compare these with any indigenous bacteria potentially containing the opd gene. The presence of the control opd-containing bacteria provides a positive control enabling the method's effectiveness to be constantly monitored.

The plate assay may employ filters impregnated with an OPA substrate. Such a substrate is either chromagenic (as in the case of parathion, paraoxon, methyl parathion, etc.) or non-chromagenic relying on a differential absorption between the substrate and product (as in the case of coumaphos).

The plate assay filters are preferably selected for their ability to bind DNA or protein. After the bacterial colonies suspected of having the opd-gene are transferred to the surface of the filter, they are lysed and fixed to the filter's surface by methods known to those of skill in the art. Next, a radioactive probe specific for the opd-gene (DNA probe) or specific for the OPA enzyme (antibody probe) is used to hybridize to the filter. The DNA probe may be any portion of the DNA sequence of the opd gene fragment which is made radioactive by ³²P incorporation during the synthesis of the probe. Alternatively, the OPA enzyme is used to produce a polyclonal antisera which, if labelled radioactively with ¹²⁵I for example, can be used to probe for OPA in the bacterial samples.

The plate assay described above may also be used in an integrated pest management system. In this application of the invention, a pest management coordinator previews a soil or crop for the presence and quantity of bacteria capable of rapidly breaking down organophosphorus compounds using the plate assay results. With this information, the pest management coordinator selects the type, quantity and formulation of pesticides to apply to a soil or crop. For example, if the coordinator finds bacteria capable of breaking down organophosphorus compounds in the soil, the coordinator may likely select a formula or pesticide that comprises no organophosphorus compounds. Alternatively, the coordinator may coformulate organophosphorus compounds which compliment each other relative to being substrates and competitive inhibitors of the OPA enzyme.

As used herein, the term "co-formulate" (or "co-formulation") refers to a combination of two or more organophosphorus pesticides where at least one pesticide

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is a preferred OPA substrate while at least one other pesticide is a competitive inhibitor of OPA.

In yet another application of the invention, the purified recombinant OPA enzyme may be used to detect the presence of trace amounts of organophosphorus compounds. This applications may be achieved in one of at least two ways. In one, OPA enzyme derived in a pure form from recombinant cells is used to pretreat aliquots of samples potentially containing organophosphorus compounds. Following this treatment, the sample and the OPA-treated aliquot are analyzed by standard pesticide residue analysis techniques. Detection of an organophosphorus compound in the untreated sample may be secondarily confirmed by selective removal or dilution by OPA in the treated sample.

In the other way, the purified recombinant OPA of the invention is used in a device wherein the enzyme is bound to a solid phase column matrix such as sepharose or DEAE cellulose and placed in a preferably small, portable column. A known volume of ambient air suspected of containing organophosphorus vapors is pumped through the column at a known rate. The exact volume of air and rate of flow can be determined based on the size of the column and the nature of the column matrix using commercially available columns and matrices. If the air contains an organophosphorus vapor sufficiently high in concentration to act as a competitive inhibitor of the enzyme, the matrix-bound OPA will be inactivated. By adding to the csm column a chromagenic substrate such as paraoxon, a measure of the amount of inactivation may be obtained. measurement of the amount of substrate converted to product (i.e. color development associated with conversion of paraoxon to p-nitrophenol) may be achieved simply by visualizing the column eluate in comparison with a range

CSM

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of color standards. The color formation may also be monitored spectrophotometrically.

This application of the invention may be used, for example, to detect air potentially contaminated with a nerve gas. Air is passed onto the column, preferably comprising a solid phase column matrix, mechanically by using a syringe and 3-way valve. After a known volume of air is passed over the column the same syringe is used to load onto the column a volume of paraoxon-containing solution buffered at about pH 8.0. After a sufficient time (i.e. time enough for color formation to occur in an unexposed column) is allowed for reaction of the bound OPA with the paraoxon, the solution is driven from the column by forcing air into the column using the syringe. color intensity generated by formation of p-nitrophenol from paraoxon is measured against a set of stable, color standards in order to ascertain the concentration of potential organophosphorus vapors in the ambient air. more intense the color, the less nerve gas vapor there is in the air sample being tested. Alternatively, the comparison may include a blank column and a column treated with a known concentration of a competitive inhibitor of OPA. In this manner, a more exact estimation of the ambient concentration of organophosphorus vapors can be made. As discussed in Example IV below, at least two typically encountered nerve gases are competitive inhibitors of paraoxon hydrolysis by OPA. OPA may be covalently coupled to column matrices by methods known to those of skill in the art.

In another application of the invention, the recombinant OPA enzyme or the recombinant opd gene itself may be used to protect certain insects against organophosphorus poisoning. For example, insects such as silk worms localized to one vicinity may be dusted with

purified recombinant OPA enzyme or with a recombinant microorganism comprising the <u>opd</u> gene. Alternatively, the recombinant OPA enzyme or <u>opd</u>-containing microorganism may be fed to a beneficial insect. Such feeding may be accomplished by adding the OPA enzyme or an <u>opd</u>-containing recombinant microoganism to the food supply such as leaves (in the case of silkworms) or to a food source of honey bees.

Beneficial insects may be protected by infecting such insects either topically or internally with opd-containing microorganisms. Most preferably, this "infection" is accomplished with microorganisms typically found in the natural flora of the insect's outer body or gut tract and transformed with a vector comprising opd. "Infection" of the insect with a naturally-associated microorganism carrying the opd-gene presents a greater likelihood that stable "infection" is achieved.

Most preferably, the opd gene itself is used to produce a transgenic insect which maintains the opd gene as a stable, inherited trait. At least two means for achieving such trangenic insects using the opd gene sequence of the invention are possible. In one means, the embryonic cells of an insect are microinjected with a vector carrying the opd gene in a transposon. transposon used must be one which can insert itself into the host insect genome while carrying with it the opd gene. The construction of the vector is such that the opd gene is placed behind a heat shock promoter of a P-element naturally associated with Drosophila. In another means, the opd gene is incorporated into a vector which causes natural viral infection of the insect host. Such a vector carrying the opd gene of the invention is injected into larvae of the host insect. This means may be accomplished

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with the baculovirus transfer vector wherein the opd gene is placed behind the polyhedron promoter of that virus.

In still another application of the invention, purified, recombinant OPA enzyme may be used to predict successful co-formulations of organophosphorus pesticides to be used in field applications where said fields are suspected or known to contain bacterial populations capable of rapidly detoxifying organophosphorus pesticides. In this manner, the pesticide applicator selects a pesticide of choice for the given application. If the chosen pesticide is also identified by the invention as being a preferred substrate of the recombinant OPA, a co-formulation pesticide is selected from the group of organophosphorus pesticides which are competitive inhibitors of OPA relative to the preferred substrate. By allowing co-formulating in this manner, the invention provides for an extended half-life for the preferred substrate pesticide and extended control of the target pest.

Experimental

The following examples illustrate various aspects of the invention. The examples should not be construed as limiting the claims.

EXAMPLE I: CLONING AND SEQUENCING OF opd

Cloning and sequencing of the <u>opd</u> gene according to this invention may be accomplished, for example, as discussed using the bacterial strains and plasmids, media and growth conditions described below.

- A. <u>Bacterial Strains and Plasmids</u>. <u>P. diminuta</u> MG is the original host of pCMS1. <u>Escherichia coli</u> strains HB101-4442 (auxotrophic for uracil and proline) and JM 103 were used as host cells for the cloning vectors, pBR322(12) and phage M13mp10 (13), respectively. Hybrid gene fusions were produced in plasmid pMC1403 and expressed in <u>E. coli</u> CQ4(28).
- B. Media and Growth Conditions. Cultures of bacteria were grown at 32°C (\underline{P} . diminuta) or 37°C (\underline{E} . coli). Nutrient medium consisted of 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl per liter (TYE). TF minimal medium (14) was used for \underline{E} . coli strains and was supplemented with uracil (50 μ g/ml), proline (25 μ g/ml), vitamin B₁ (0.01%), Casamino Acids (0.1%), glucose (0.2%), and antibiotics (25 to 50 μ g/ml) as required.
- C. <u>Isolation of Plasmid DNA</u>. Standard protocols for the isolation of DNA from <u>E</u>. <u>coli</u> for plasmid (15) or phage (13) have been described. Isolation of predominantly covalently closed circular plasmid DNA from <u>P</u>. <u>diminuta</u> was accomplished via a mild lysis procedure modified from that of Berns and Thomas (16).
- Plasmid. The PstI restriction fragments of pCMSl were inserted into pBR322, inactivating the ampicillin gene (Focus 5:3, Bethesda Research Laboratories [BRL], Gaithersburg, Md., 1983). The resulting recombinant plasmids were used to transform competent HB101-4442, and tetracycline-resistant (Tc^r) colonies were selected and evaluated for ampicillin sensitivity (Ap^S). The plasmid structure of selected Tc^r Ap^S transformants were determined, and clones representing the different inserts were analyzed for activity.

The 1.3-kb PstI insert of pBR322-038 was excised from its vector, purified by preparative agarose gel electrophoresis using a modified freeze-squeeze phenol procedure (S. A. Benson, Biotechniques March/April:66-67, 1984), and subsequently introduced into the multiple cloning site of M13mp10. The resulting recombinant molecules were transformed into competent E. Coli JM103 cells, and clear-plaque isolates were selected. All subsequent manipulations of viral recombinant DNAs were performed according to the methodology of the Bethesda Research Laboratories (BRL) "M13 Cloning/Dideoxy Sequencing Manual." A variety of 5' and 3' deletions of pBR322-038 were constructed, using various restriction sites surrounding the opd gene (BamHI, AvaI, NruI, SalI, SphI). In addition, 3' exonuclease III deletions were used to identify gene boundaries.

Dideoxy sequencing was accomplished by the method of Sanger as detailed in the BRL "M13 Cloning/Dideoxy Sequencing Manual." In cases where GC compaction was evident, reverse transcriptase as well as the Klenow fragment of DNA polymerase was used (BRL, manufacturer's protocols). Oligonucleotide primers were synthesized using phosphoramidite chemistry with an Applied Biosystems Synthesizer according to the manufacturer's recommendations.

The 5' region of the <u>opd</u> gene was subcloned into the β -galactosidase gene for the purposes of producing a <u>lac</u>Z fusion polypeptide. The 1.3-kb opd fragment was restricted with <u>Ava</u>I; the staggered restriction fragment was end-filled and ligated into the 5' <u>Sma</u>I cloning site of the <u>lac</u>Z fragment of pMCl403(28). This hybrid genetic construction was then transformed into <u>E</u>. <u>coli</u> CQ4(17).

E. OPA Enzyme Assay. Routine analysis of parathion hydrolysis in whole cells was accomplished by suspending cultures in 10 mM Tris hydrochloride (pH 8.0) containing

1.0 mM sodium EDTA (TE buffer). Cell-free extracts were assayed using sonicated extracts in 0.5 ml of TE buffer. The suspended cells or cell extracts were incubated with 10 μ l of substrate (100 μ g of parathion in 10% methanol), and p-nitrophenol production was monitored at a wavelength of 400 nm. To induce the gene under <u>lac</u> control, 1.0 μ mol of isopropyl- β -D-thiogalactopyranoside (Sigma) per ml was added to the culture media.

F. Column Chromatography, Affinity Chromatography, and Protein Sequencing. P. diminuta cells from a 200-liter fermentation were harvested by a continuous-flow centrifuge and suspended in 2.0 liters of 1.0 M NaCl. Samples of this suspension were agitated in a Waring blender for 30 s, and the resulting suspension (5.0 ml) were sonicated, treated with 0.1% Triton X-100, and stirred at room temperature for 2 h before chromatography.

The molecular weight of the native enzyme was determined by ascending Sephadex G-200 chromatography in the presence of 50 mM CHES buffer [2-(N-cyclohexyl-amino)ethanesulfonic acid (pH 9.0)] at 4°C. Enzymatic activity was located by introducing 50- μ l alliquots of column fractions (2.0 ml) into a reaction volume of 0.8 ml containing 0.2 mM paraoxon and 50 mM CHES buffer (pH 9.0).

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Purification of hybrid β-galactosidase proteins encoding the 5' region of the opd gene was achieved by immunoaffinity chromatography and preparative gel electrophoresis. Gas-phase sequencing of the purified fusion polypeptide (Applied Biosystems 470A Sequencer, Applied Biosystems 120A On-Line-PTH Analyzer, was accomplished by the methods of Hewick, et al.(18).

G. <u>Cloning of pCMS1 into PBR322</u>. The entire DNA from the degradative plasmid was digested with <u>PstI</u>

(generating fragments of approximately 18.5, 17.3, 5.3, 4.3, 1.7, 1.6, 1.3, and 0.8 kb) and was subcloned into pBR322 within that vector's ampicillin gene. Cell-free lysates of Ap^S clones selected from the Tc^r transformants of E. coli HB101-4442 were tested for activity. A single-colony isolate was selected for its ability to hydrolyze parathion, and the expected phenotype (Tc^r Ap^S; auxotrophy for uracil and proline; parathion hydrolysis) was verified. A 5.6kb, CsCl-purified plasmid isolated from this strain was used to transform competent HB_101-4442 cells, regenerating the phenotype and demonstrating that the hydrolytic activity is mediated by the recombinant plasmid.

Insertion of the 1.3-kb PstI fragment into the multiple cloning site of Ml3mpl0 produced an opd-encoding phage (Ml3mpl0-038/008) possessing an inducible (isopropyl- β -D-thiogalactopyranoside) whole-cell activity in E. coli JMl03. This phage may be used in hybridization studies ("C-tests") to select other isolates which possess similarly sized insertions but lacked activity.

H. <u>Nucleotide Sequencing</u>. Dideoxy sequencing along both strands of the <u>opd</u> gene revealed a potential translational reading frame (Fig. 1). Oligonucleotide primers were constructed for the purposes of sequencing regions lacking convenient restriction sites. In all cases, these primers were selected to efficiently promote DNA synthesis.

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The open reading frame begins 12 base pairs from the 5' PstI site. A potential start site (ATG) was located 17 codons into the open reading frame. This codon appeared to be a candidate for the translational start since it is preceded by an AAGCAA sequence 15 base pairs upstream; the sequence and spacing are in good agreement with known Pseudomonas ribsomal binding sites (19). An N-terminal

deletion of some of the sequence prior to the ATG start codon is possible without complete loss of activity. In addition, several potential Rho-dependent terminator structures ranging in free energy of association from - 12.6 to -15.4 kcal/mol (ca. -52.7 to -64.4 kJ/mol) were located 3' of the open reading frame.

- I. Amino Acid Sequencing of Fusion Polypeptides. When a fusion protein is constructed between the 5' region of the opd gene and the lac gene at the AvaI-SmaI site, a hybrid polypeptide can be recovered, purified, and subjected to amino acid sequencing. This confirmed the reading frame.
- J. <u>Subcloning regional deletions</u>. Figure 2 summarizes results obtained with various subclones of the 1.3-kb fragment containing the <u>opd</u> gene. Deletions outside the putative coding region remain active when the sequence is properly oriented for expression from the <u>lac</u>Z promoter. If the orientation is reversed or if deletions are made within the putative coding region, activity is eliminated. In particular, it was possible to remove the C-terminal <u>Bam</u>HI <u>Pst</u>I fragment without significant loss of activity.

EXAMPLE II: HETEROLOGOUS EXPRESSION IN INSECT TISSUE CULTURE

Heterologous expression of <u>opd</u> in insect tissue culture may be obtained according to this invention as described in the example below.

A. <u>Molecular Manipulations of Recombinant DNA</u>

<u>Vectors.</u> Standard recombinant DNA techniques were employed (20), using enzymes purchased from either

Bethesda Research Laboratories, Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, IN. A BamHI restriction fragment, containing the entire opd open reading frame, 62 bp of the 5' flanking sequence, and 17 bp derived from the polyclonal region of M13mpl0, is isolated from the RF DNA of clone mp10-008. This 1170 bp fragment was subcloned into the BamHI site of the tac promoter vector pDR540, obtained from Pharmacia, Inc., Piscataway, N.J., and the resulting construction was designated pLH540opd was transformed into the lacI- E. coli strain MC4100 (17). This same BamHI fragment was subsequently isolated from pLH540opd and cloned into the BamHI site of the baculovirus transfer vector pVL941 (21). The resulting construction was designated pLH1170opd and was a derivative of pAC311 in which the polyhedron ATG start has been mutated by site-directed mutagenesis to an ATT triplet. Translation of a foreign protein expressed from the polyhedron promoter in pLH1170opd is thus initiated at the first ATG codon in the foreign gene's ORF, and a non-fused protein is produced. Expression of the native opd sequence is under the control of the baculoviral polyhedron promoter (hed-promoter).

B. Production of recombinant virus. Spodoptera frugiperda (fall armyworm) sf9 cells (22) were cotransfected with wild-type (AcMNPV) viral DNA and pVL941-29 by a modification of the calcium phosphate precipitation technique. The transfected cultures were incubated for 5 days in TMN-FH media (23) supplemented with 10% (v/v) fetal calf serum at 27°, after which the supernatants were removed from each well and saved as viral stocks. The cells in each well were lysed by the addition of 200 ul of .5N sodium hydroxide. The lysates were neutralized by the addition of 20 ul of 10 M ammonium acetate to each sample, and each lysate was then spotted onto a nitrocellulose filter. The filter was baked for 2

hrs at 80°, and the lysates were screened by hybridization with a nick-translated, ³²P-labelled, <u>opd</u>-specific probe. Clones were selected for further purification and analysis on the basis of their strong hybridization signal. A second transfection was performed as above, and recombinant plaques were transferred to a 96-well plate. After 2 days at 27°C, the infected wells were assayed directly for expression of the <u>opd</u> gene product by the addition of ~100 ul of 3.6mM paraoxon (pH 7) to each well. The plate was incubated for 2 hrs at 27°C. <u>Opd+</u> recombinants were detected by the enzymatic release of p-nitrophenol, producing an intense yellow color in <u>opd+</u> wells. Isolates were selected for further purification and analysis.

C. Relative enzymatic activity of bacterial and baculoviral opd constructions. Table I summarizes an examination of the relative expression of the opd by measuring the activity of its enzymatic product. hydrolysis of paraoxon under standard conditions was used for comparative purposes and the enzymatic activity is expressed as 1 umole of paraoxon hydrolyzed to diethylphosphate and p-nitrophenol per milligram per minute (units/mg protein). Activity was measured at 25°C in a Gilford Response Spectrophotometer or Gilford model 260 spectrophotometer ($\xi_{400} = 17,000 \text{ M}^{-1} \text{cm}^{-1}$). of expression in E. coli strains with various promoters (lac, tac, bla and other constructions at 37°C were disappointing and never exceeded 3-4% of that obtained in the native Pseudomonas diminuta MG source). This difference is even more dramatic than apparent since the expression from the lacP was attempted on high copy plasmids (pBR322-based constructions) and M13constructions (best expression reported in Table 1). use of the tacP of the expression vector pDR540 does not

produce increased expression under conditions of IPTG-induced expression in the MC4100 \underline{E} . coli host background.

When expression studies are shifted from bacterial systems to baculoviral transfection in insect tissue culture, further production can be realized. In Sf9 cell cultures under expression of the hedP promoter of polyhedron from Autographa californica, a nuclear polyhedrosis virus, the enzymatic activity is increased to 10-15 units per mg protein in the primary culture. This permits the complete purification of the enzyme. Expression requires transfection with the wild-type virus for complete infection and development.

EXAMPLE III PURIFICATION OF OPA & SUBSTRATE SPECIFICITY

Purification of the recombinant OPA from insect tissue culture may be achieved according to this invention as described in the example below.

A. General—Enzymatic activity was measured by monitoring the absorbance at 400 nm as 0.75 mM paraoxon was hydrolyzed to diethylphosphate and p-nitrophenol ($\epsilon_{400} = 17,000 \; \text{M}^{-1} \text{cm}^{-1}$) in 150 nM CHES, pH 9.0, buffer using a Gilford model 260 spectrophotometer regulated at 25&°C. One unit of activity was defined as the hydrolysis of 1 µmole of paraoxon/min. Protein concentration in crude samples was determined by measuring the absorbance at 280 nm or by the bicinchoninic acid assay method developed by Smith et al. (24) (Pierce Chemical Co.) with bovine serum albumin as a standard. Denaturing polyacrylamine gel electrophoresis was carried out by the method of Laemmli (25) and protein was visualized by silver staining according to the method of Wray, et al. (26).

В. Purification of the enzyme--The enzyme from Pseudomonas diminuta was purified from sf9 cells (fall armyworm) infected with the recombinant baculovirus (pVL941-29) containing the opd gene. The cells were infected at a cell density of 2-2.5 x 10⁶ cells/mL with 0.2 mL virus/mL cells. The virus generally should have a titer of 1×10^8 (plaque forming units)/mL. infection was allowed to proceed at 27°C for four days before harvesting the cells by centrifugation at 6100 \times g for 30 min at 4°C. All subsequent steps in the purification were carried out at 4°C. The baculovirus infected sf9 cells (5-6 q/L of cell culture) were resuspended in 50 mM triethanoloamine pH 9.0 buffer containing 0.1 mM χ nCl₂ (buffer A) and gently stirred for one hour. Cell lysis was achieved by 5 sec pulsedsonication for 5 min at a medium power setting using a Heat Systems - Ultrasonics, Inc. model W830 ultrasonic processor with a macro-probe tip. This suspension was centrifuged at 25,000 x g for 30 min. The supernatant fluid was decanted and the cells were resuspended in buffer A and centrifuged as before. This supernatant fluid is combined with the previous supernatant fraction. DEAE-cellulose (DE-52, Whatman), washed and equilibrated in buffer A, was added to the combined supernatant fractions at a concentration of 1 mL settled gel per 500 mg protein. This slurry was swirled for 30 min and filtered through a coarse scintered glass funnel, retaining the filtered solution for application to a 2.5 x 48 cm Green A dye matrix column (Amicon Corp.) equilibrated in buffer A. The enzyme was applied at a rate of 1 mL/min. The column was extensively washed with buffer A before initiating a 800 mL,0-700 mM KCl gradient in buffer A at a rate of 1.0 mL/min. The fractions containing enzyme activity are pooled and loaded onto a phenyl sepharose column (2.5 x 15 cm) eguilibrated in buffer A containing 700 mM KCl. After loading at a rate

of 1 mL/min, the column was thoroughly washed with buffer A. The enzyme was eluted in a 800 mL 0-60% ethylene glycol gradient in buffer A at 1.0 mL/min. The fractions containing the enzyme were pooled and aliquots of 30 mL were loaded on a 2.5 x 90 cm G-75 Sephadex column equilibrated in buffer A and chromatographed.

C. Purification - Table 2 summarizes the results of a typical purification procedure. Elution of the enzyme with 700 mM KCl resulted in a 20-fold purification. Moreover, the relatively high salt concentration aided in the hydrophobic interaction between the enzyme and the phenyl sepharose media used in the subsequent column. introduction of ethylene glycol into the phenyl sepharose column allowed elution of the enzyme without the denaturing side effects often observed with other organic solvents. This purification step provided an additional 16-fold purification. The enzyme could be further purified by gel filtration to give a homogeneous preparation. From approximately 8 g of pVL941-29 infected sf9 cells, approximately 2.7 mg of homogeneous enzyme were obtained with an overall yield of 75% after a 1500-fold purification.

Table 2: Purification of Phosphotriesterase

Step	Volume (mL)	Activity (mmol/min)(mg)	Protein	Specific Activity (units/mg)	Fold	Yield
Sonicate	226	11400	5400	2.1	1.0	100
DE-52	450	11800	1550	7.6	3.6	104
Green A	124	9390	62.4	150	71	82
Phenyl Sepharose	117	10100	4.2	2400	1140	89
G-75	390	8520	2.7	3200	1500	75

- Substrate Specificity Organophosphorus compounds were evaluated as substrates at pH 9 in 100 mM CHES buffer. The reactions were monitored spectrophotometrically, either in the UV region or within the visible spectrum depending upon the characteristics of the substrate. Pesticides such as 0,0-diethyl-0-[3-chloro-4methyl-2-oxo-2H-lbenzopyran-7-yl]phosphorothioate (coumaphos), 0,0-dimethyl-p-cyanophenyl phophorothioate (cyanophos), 0,0-diethyl-0-[2-isopropyl-4-methyl-6pyrimidyl] phosphorothioate (diazinon), 0,0-diethyl 0-[3,5,6-trichloro-2-pyridyl] phosphorothioate (dursban), O,O-diethyl [p-(methylsulfinyl) phenyl] phosphorothioate (fensulfothion), 0,0-diethyl-0-p-nitrophenyl phosphorothioate (parathion), and 0,0-dimethyl-0-pnitrophenyl phosphorothioate (methyl parathion) were purchased from Chem Service, Inc., West Chester, Pa. limited solubility of these compounds necessitated the addition of 10% methanol to the reaction mixtures.
- E. Substrate Specificity There was an extensive set of commercially used organophosphate pesticides that are hydrolyzed by the recombinant OPA enzyme (Table 3). The OPA enzyme from Pseudomonas diminuta will hydrolyze many of the commonly used organophosphorus insecticides in addition to paraoxon. Replacement of the phosphoryl oxygen with a sulfur increases the K_m but reduces V_{max} . Substitution of methoxy for ethoxy groups produces substrates with higher K_m values and reduced catalytic

rates. The size of the leaving group appears to be relatively unimportant since coumaphos was hydrolyzed at a rate comparable with parathion. This suggests that there are probably few molecular interactions between the enzyme and the leaving group. The dominant factor in the rate of substrate hydrolysis was stabilization of the anionic product (This aspect is further described in Lewis, et al. 1988. Biochemistry 27: 1591-1597).

The experimentally determined value for k_{cat} and k_{cat}/K_m with paraoxon as a substrate are substantial. The k_{cat}/K_m of 4 x 10⁷ M-¹ s-¹ was very close to the diffusion controlled limit of 10⁸ - 10⁹ M-¹ s-¹. Thus, the enzyme activity of this protein was quite respectable.

Table 3: Kinetic Constants for the Hydrolysis of Organophosphorus Insecticides

•	Structure	Common Name	wavelength (nm)	K _m (mM)	V,**	(V.K) _{rei}
Ε	0 E:0 - P - 0 - NO ₂	paraoxon	400	0.09	100	100
E	S C1 C1 C1 E:0 N C1	dursban	276	0.11	0.08	0.07
E: ~	S SO - P - O - NO ₂	parathion	400	0.24	30	11.25
E	S CI 10 - P - 0 CI	coumaphos O	348	0.39	29	6.70
€:	S Me C - P - C N N N N Pr	ciazinon	228	0.45	8.4	1.68
E :	:0 - ; - 0 - ; - s - ; - s - ; - ; - ; - ; - ; - ;	Me fensulfothion	284	0.46	3.2	0.63
i	S NO ₂ NO ₂	methyl-parath	icn 400	0.84	2.4	0.25
ŧ	S 1.1e - ? - O - CN	cyanophos	274	2.1	75	0.32

F. Amino Acid Composition - The amino acid composition was determined for a homogeneous preparation of OPA enzyme and compared favorably with the predicted composition from the DNA sequence of the opd gene. The N-terminus could not be sequenced, apparently due to some terminal modification of the protein.

EXAMPLE IV DETOXIFICATION OF NERVE AGENTS

The organophosphorus-degrading genes (opd) isolated from plasmids of Pseudomonas diminuta and Flavobacterium encode identical Organophosphorus Acid Anhydrases (EC 3.1.3.-) which are capable of hydrolyzing a wide spectrum of insect and mammalian neurotoxins. The Pseudomonas enzyme can be purified following expression from a recombinant baculoviral vector in insect tissue culture of the Fall Armyworm, Spodoptera frugiperda (Sf9 cells). Purified enzyme preparations have been shown to be able to detoxify a number of structurally related acetylcholinesterase inhibitors including the organophosphorofluoridate nerve agents, sarin and soman. This was the first recombinant DNA construction capable of degrading these potent nerve gases. This enzyme was capable of degrading the common organophosphorus insecticide, paraoxon, at rates exceeding 2 \times 10⁷ M^{-1} $(mole\ enzyme)^{-1}$ which are equivalent to the most. catalytically efficient enzymes observed in nature. purified enzyme preparations are capable of detoxifying O,O-diisopropyl phosphorofluoridate (DFP), a less toxic model mammalian neurotoxin, and 1,2-dimethylpropylmethylphosphorofluoridate (sarin) at equivalent rates (50-60 molecules per molecule of enzyme per second). addition, the enzyme can hydrolyze 1, 2, 2 trimethylpropylmethyl-phosphorofluoridate (soman) at

approximately 10% of the rate of Sarin. The breadth of substrate utility and the efficiency for the hydrolysis exceeds the known abilities of other prokaryotic and eukaryotic organophosphorus acid anhydrases and it is clear that this detoxification profile was due to a single enzyme rather than a family of related, substrate-limited proteins.

EXAMPLE V PROTECTION OF INSECTS WITH OPA

Insects may be protected according to this invention using recombinant opd-containing microorganisms or purified OPA in a manner similar to the example below. The example describes use of crude enzyme extract only.

- A. Wild type <u>Drosophila melanogaster</u> propagated by standard methods (27) by using a medium containing per liter: 100 g glucose, 10 g agar, 100 g yeast and 3 g p-hydroxybenzoate as a fungistat. Adult flies of approximately the same age were used for each assay to avoid any fluctuation in pesticide susceptibility due to age differences. Flies were anesthetized with ether before being transferred and checked for their full recovery before exposure to the pesticide in testing vials.
- B. <u>Flavobacterium</u> sp. (ATCC #27551) were grown in nutrient broth containing per liter: 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride. Overnight cultures were harvested by centrifugation, and the cell pellet was washed and dried three times with acetone. The hard pellet was then pulverized with mortar and pestle. The resulting dried cell powder can be and was stored at 4°C for several months. For the bioassay, a fresh OPA

enzyme solution was made by dissolving 0.05 g of dry cell powder in 5 ml of Tris-HCL buffer (10 mM, pH-8.5).

- C. Small double filter papers (Whatman 984H, 2.4 cm size) were impregnated with exact amounts of pesticide solution by means of a microsyringe. The solvent (2, 2, 4-trimethylpentane, hexane, acetone, or ethyl acetate) was allowed to evaporate for 30 min. Control filters impregnated with the same volume of solvent showed no acute affect and indicated that 30 min. was sufficient to allow evaporation of all of the solvent.
- D. A fixed amount of either PTE enzyme solution or buffer was also impregnated on the filters and incubated at 37°C for 30 min. to allow pesticide degradation. At the end of the incubation, exactly 10 flies along with a few drops of sugar or honey water were added into each testing vial containing the filters. The vials were allowed to stand at room temperature overnight (between 16 to 20 h) and the percent fly survival in each vial was determined by visual inspection.

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E. Parathion was one of the more commonly used pesticides and was also a good substrate for the OPA enzyme. Results in Table 4 show that all the flies were killed by parathion in the test vials where $\frac{OPA}{PTE}$ was not added. Even at the lowest amount of parathion applied (1.2 μ g) no fly survived overnight. In contrast, all the test flies survived where the pesticide-impregnated filters were treated with OPA (0.87 mg) prior to the fly test.

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Table 4. Crude Enzyme Effect on Certain Organophosphorus Pesticides.

		Crude	Percent
Pesticide	(µg)	enzyme (mg)	survival
Parathion	1.2	0	0
	1.2	0.87	100
	12.4	0	0
	12.4	0.87	100
	41.1	0	0
	41.1	0.87	100
	82.6	0	0
	82.6	0.87	50
	82.6	1.74	100
	124	0.87	0
Diazinon	11.0	0	0
	11.0	0.87	100
	22.0	0	0
	22.0	0.87	100

When the amount of parathion was increased to 82.6µg, only 50% of the flies survived in OPA-treated test vials, and suggested that more enzyme may be needed for detoxifying all the parathion. This was found to be true when the amount of OPA was doubled, as also shown in Table 4. These results indicated that to get a conclusive result, it was important not to have an excessive amount of pesticide so that the amount of OPA becomes the limiting factor. This can be achieved by performing multiple tests using different dilutions of the same sample. Essentially the same results were obtained with diazinon (Table 4).

F. Since certain organophosphorus pesticides, and other insecticides such as carbamates are not degraded by the OPA enzyme, one would predict that the survival in the bioassay should not be affected by the enzyme treatment. Results shown in Table 5 for certain organophosphorus and carbamates pesticides, respectively, indicate that for a wide range of pesticide concentrations, there was no difference in survival between enzyme-treated and untreated samples. This demonstrates the basis of this modified fly test which was to rely on the specificity of OPA toward detoxification of certain pesticides.

Table 5. Crude Enzyme Effect on Certain Organophosphorus Pesticides and Carbamate Pesticides

	•	Crude	Percent
<u>Pesticide</u>	(µg)	enzyme (mg)	survival
Fenthion	1.5	0	0
	1.5	0.87	0
	7.7	0	0
	7.7	0.87	0
	46.0	0	0
	46.0	0.87	0
Carbofuran	1.0	0	100
	1.0	0.87	100
	7.6	0	2.0
	7.6	0.87	10
	11.5	0	. 0
	11.5	0.87	0
	22.9	0	0
	22.9	0.87	0
Aldicarb	9.5	0	0
	9.5	0.87	0

EXAMPLE VI PROTECTION OF INSECTS WITH RECOMBINANT opd GENE

A recombinant baculovirus containing the <u>opd</u> gene was constructed using recombinant DNA techniques as described previously. The open reading frame from vector pDR540-1 was inserted into the baculovirus vector pVL941 (21).

To assess whether the expression of a functional OPA enzyme within living insects will protect against the lethal effects of an insecticide, the recombinant virus was injected directly into the larval stage of the fall army worms. The caterpillars were injected late in the third instar by taking up 5 μ L of 2 x $10^8/m$ L pVL941-29 into a fine capillary needle and injecting it into the hemolyph through one of the prolegs. After a relatively short lag phase, activity was detected in the caterpillar when paraoxon was used as a substrate. For the first four days after injection, the enzyme activity increased about an order of magnitude every 24 hours until it reached a maximum of approximately 11 units of paraoxon hydrolyzing activity per caterpillar. No enzyme activity ($<5 \times 10^{-5}$ units/caterpillar) was detected in uninfected caterpillars or caterpillars infected with the wild-type baculovirus.

The effect of the insecticide paraoxon can be determined on both the infected and uninfected caterpillars. Shown in Figure 4 is a plot of mortality

versus the amount of paraoxon that can be applied directly to the caterpillars. This graph demonstrates that those caterpillars containing a functional OPA are resistant to all but the highest concentration of paraoxon that was applied. The LD $_{50}$ for the pVL941-29 infected caterpillars was calculated to be 2000 \pm xx μ while the control group csm (containing a mixture of uninfected and pAC311 infected catepillars) had an LD $_{50}$ of $9\pm$ xx μ g. Thus, the lethal csm dose increased by a factor of at least 220 due to the presence of the opd gene. In the moth stage the LD50 was found to be 1 μ g of paraoxon.

The results presented above demonstrated that resistance to paraoxon and other pesticides can be induced in insects by expression of an enzyme that was known to efficiently hydrolyze these molecules to nontoxic products. It should, therefore, be feasible to construct alternative systems involving the incorporation of the recombinant opd gene into other insect species.

EXAMPLE VII CHROMAGENIC ASSAY USING opd+ CONTROLS

- A. <u>Bacterial Strains and Media Pseudomonas diminuta</u> MG was the original host of pCMS1. Cultures were grown at 32°C on nutrient media consisting of 10 g Bacto-tryptone, 10 g Bacto-yeast extract, and 5 g of NaCl per liter (TYE). <u>Ps. diminuta</u> strains were maintained as 40% glycerol stocks at -70°C.
- B. <u>Plate Assay Technique</u> Filter pads (8.5 cm) of Whatman No. 1 filter paper were treated by spraying evenly with a 2-3 ml volume of a 10mg/ml solution of technical parathion (Monsanto) in methanol. The pads were forced-air dried and stored in the dark at room temperature until used.

Pesticide-impregnated pads were applied to the surface of TYE plates grown at 32°C and containing colonies which were allowed to grow to a diameter of 2-4 mm. The colony populations of each plate were lifted off the surface onto the treated pad. Parathion hydrolysis was permitted to continue for 30 min at 37°C in a humidified incubator at pH 9.0. Single colonies of non-degrading strains (opd-) were identified from among parathion-degrading strains (opd+) on plates by the appearance of the yellow product, 4-nitrophenol. Conversely, rare opd+ isolates were selected from among numerous opd- colonies. In all cases, the pads were marked appropriately to allow for further reference and the master plate was reincubated for regeneration of the original colonies.

C. <u>UV-Photography and Eclipsing Method</u> The described method relies on the release of a chromogenic product (4-nitrophenol) and the absorption of ultraviolet irradiation (maximum absorbance = 400 nm, molar extinction coefficient = 1.88 x 10⁻⁴). UV-enhanced photography of filter pads was accomplished by first photographing (tungsten filament) the master plate prior to lifting off the colonies onto the treated pad. A treated pad was used to lift the master colonies off the plate and after sufficient time has been allowed for 4-nitrophenol development, a photograph is taken, using a combination of short (254 nm) and longwave (366 nm) ultraviolet irradiation.

The negatives of the two exposures are aligned in such a way as to precisely overlay the colonies of the lift negative above those same colonies on the master plate; the dark 4-nitrophenol producing (UV-absorbing) colonies of the pad lift eclipsed the bright, white colonies of the original master plate.

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D. Results. Figure 5a shows a TYE plate containing approximately 500 colonies of Pseudomonas diminuta photographed as described. It was possible to identify opd-colonies sufficiently separated from surrounding opd+colonies. Such a colony was indicated in Figure 5a.

However, in order to quantitate large numbers of closely packed colonies, the UV-absorbing characteristic of 4-nitrophenol were used to produce a black and white image of the developed (4-nitro-phenol coloration) filter lift. Figure 5b shows the results of such a photograph of the filter lift produced when the master plate in Figure 5a was blotted with a parathion-impregnated filter pad. The colony previously identified by visualizing 4-nitrophenol development under white light is seen as a non-absorbing spot in contrast with the majority of opd+colonies which appear black using this photographic method.

Figure 5c demonstrates the photographic enhancement which permits the identification of rare colonies among colonial masses which no longer possess the phosphotriesterase activity. This image was produced as described by eclipsing the two negatives of the photographs in Figures 5a and 5b. The resulting image indicated at least two additional opd- colonies seen here as half moon-shapes in addition to the one originally identified in Figure 5a and b. Using this technique, it was possible to quantitate and isolate opd- derivatives from the parental opd+ Pseudomonas diminuta strain.

As a test of the sensitivity of the method for identification of rare opd+ colonies among many opd-colonies, opd+ cells of Pseudomonas diminuta MG were mixed with cells of an opd- derivative of that strain. Ratios of positive to negative phenotypes vary from 1/100 to

esm es m 1/100,000. At all dilutions, the rare <u>opd+</u> colonies were visible. Plates containing as many as 10,000 colonies were readily screened with a 95% efficiency.

EXAMPLE VIII DETECTION OF <u>opd</u> - CONTAINING BACTERIA USING DNA PROBES

- Subcloning Test Fragments. The nonidentical nature of the two separately isolated plasmids was demonstrated by using a pair of PstI fragments from the P. diminuta plasmid (pCMS1) as probes against the plasmid DNA of the Flavobacterium sp. (Fig. 6). The cloning of the two PstI fragments (C" and D) from pCMS1 has been described previously (Example I). The C" fragment from the Pseudomonas plasmid (1,326 bp) containing the opd gene was shown by sequence analysis to be identical to the samesize fragment from the Flavobacterium plasmid. A second PstI fragment (D) of approximately 900 bp was chosen as a probe since it was separated from the region containing the known homology by approximately 22kb, as estimated by a preliminary restriction digest map of the Pseudomonas plasmid. For all of the hybridization studies, the methods of Southern were used (29).
- B. Identity of opd Fragments. Figure 7 demonstrates the strong hybridization of both Pseudomonas and Flavobacterium plasmid DNAs with the 1,325-bp (C") fragment containing the opd gene sequence. The PstI-digested plasmids differed considerably in their restriction profiles (Fig. 7A). There appears to be a single plasmid in the Flavobacterium strain, although it was present in several forms. Upon restriction, a single hybridizing band was observed for each of the two plasmid sources of the gene (Fig. 7B), and the overall restriction

endonuclease pattern was similar to that observed for the isolated plasmid.

When the 900-bp fragment (D) was used as a probe against both plasmid DNAs (Fig. 8A and B), it hybridized to DNA in the control (PstI-digested pCMS1) and the unrestricted Pseudomonas plasmid. However, the 900 bp fragment failed to hybridize to either the native or the restricted plasmid DNA from Flavobacterium sp. These results were consistent with the restriction site data and reiterated the dissimilarity of the two plasmids.

C. Screening Other Strains for opd using DNA probes.

Table 3 summarizes studies in which 8 different bacterial systems were evaluated for sequence similarity to the opd gene sequence in order to evaluate whether those bacterial systems carry hybridizing DNA sequences and compare paraoxonase activity with DFPase activity. It was possible with the present invention to screen microorganisms in order to find out whether they have DNA sequences that are similar to opd.

Table 6.

Bacteria Strains Possessing
OPA Anhydrase Activity

Strain	DFPase(mM/min)	Paraoxonase(mM/min
B. subtilus globigii	0.075X10 ⁻³	0.036
P. acidovorans	ND	0.059
Flavobacterium sp. ATCC 27551	29.5X10 ⁻³	4.88
E. coli JM109	0.006x10 ⁻³	0.23
E. coli JM103/opd	0.05x10 ⁻³	0.42
P. diminuta PD3 ⁺	61.7×10^{-3}	1.34
reaction conditions:	50mM BTP pH 7.2, 400 100um ZnCl2, 400uM M	

The principle of the invention and the best mode contemplated for applying that principle have been described. It was to be understood that the foregoing was illustrative only and that other means and techniques can be employed without departing from the true scope of the invention defined in the following claims.

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WE CLAIM:

 A cloned bacterial organophosphorus acid anhydrase gene fragment comprising the DNA coding sequence:

```
CTSCAGGGTGAGTGSGCACCAGTGSGTSGAAGGTAGGCAATGSGAAGGGGGGGAGG
ATS CAA AGG AGA AGG STT STG STG AAG TGT SGS GGS SGA GGA ACT STS GTG SGC
sec gin the arg arg wal wal leu lys ser ala ala ala gly the leu leu gly
GGC CTG SCT SGG TGC SCS ACS TGG CTG SAT CSA TCS SCA CAG SCS ATC SGA TCA
gly leu ala gly cys ala thr trp leu asp art ser ala gin ala lle gly ser
ATA COT GCG COT CCT ATC ACA ATC TOT GAA GCG GOT TOT ACA CTG ACT TAC GAG lie arg ala arg pro lie the lie ser glu ala gly pne the leu the his glu
GAC ATC THE SEC AGE TOO GEA HER HTT THE COT HET THE COA HAR THE THE HER AND THE CYS GIV HER HER HIS GIV PRE LEW AND ALL THE PRO GIV PRE PRE GIV
AGC COC AAA GCT CTA GCC GAA AAG GCT GTG AGA GGA TTG CGC GCC AGA GCG GCT ser arg lys ala leu ala giu lys ala val arg giy leu arg ala arg ala ala
SGC STS COA ACS ATT GTC SAT STS TCS ACT TTC SAT ATC SGT CSC SAC STC AGT gly val arg thr ile val asp val ser thr one asp ile gly arg asp val ser
TTA TTG GCC GAG GTT TGG GGG GGT GCC GAC GTT GAT ATC GTG GGG GGG ACC GGC leu leu ala glu vai ser arg ala ala asp vai his ile vai ala ala thr gly
THE THE THE GAC CES CEA CTT TOS ATS ESA THE AGE TAT STA GAS SAA CTT ACA leu try pne amp pro pro leu mer set arg leu arg tyr wal glu feu ter
cad the the one con dad ant cal har ode and dad ace oda ant ago des gin one one leu and glu lie gin byt gly lie glu asp the gly lie and ala
one art are had one dec acc acc and one and occ occ fire can gad tha one gly lie lie lys wal als the the gly lys als the pro one glm glu leu wal
THE AND GOS GOD GOD GOD AGO THE GOD AGO SET STEET GOD AGO ACT CAG
law lys ala ala are all ser law ala the gly val per val the the bis
ACC SCA SCA ACT CAG COC SAT SOT SAG CSA SGC AGG CCS CCA TTT TTS AGT CCS
the sia sia ser gin arg asp gly giu arg gly seg pro pro pae leu ser pro
ANG CTT SNG CCC TON CGG GTT TOT ACT SOT SNC AGC SNT SNT ACT SNC SAT TTO
Lys lau glu pro ser arg wal cys lie gly his ser asp asp the asp asp lau
AGE TAT THE ACE SEE CTS CTS CSC SGA TAC THE ATO SET TA GAC TAC ATO SEE THE LEW THE SIA LEW LEW ATT THE LIE THE SIA LEW LEW ATT THE LIE THE SIA LEW LEW ATT THE
the AGT Ses ATT GGT CTA SAA SAT AAT SES AGT SCA TCA SES STE STS GGC ATC his ser ala ile gly leu glu asp asn ala ser sia ser pro leu leu gly lie
con now now call ack cod son one one and also son one and sale call soo take any sen unp gin the any all les les lies lies all less lies any gin giv type
ATS ANA CHA ATC OTC STT TOS ANT SAC TSS CTS TTC SGS TTT TOS AGC TAT STC set lys gin the lew wal ser asm asp trp lew pne gly pne ser ser tyr wal
ACC AAC ATC ATG GAC GTG ATG GAT CGC GTG AAC GGC GAC GGG ATG GGC TTC ATT
thr ash the met asp wal met asp are val ash pro asp gly met ala pae ile
cm cm and one are cm mm the end and and one fee the and and che fee pro leu and val ile pro pne tyr glu and and ala sen his and lys and cys
CAG SCA TCA CTO TGA

gin ala ser leu

CTARCOSOGOSOTTOTOTOTOACCAACTTSCCGTGCATGACGCCATCTSGATCCTTTCACGCAGGGGGC

CTARCOSOTGCGGTTAAAGCGAACGATGAAGTGGCAATGATAGGCATGTTCAATGTAATCAGGA

CTGCACCTTCAAAAGCCGGTSGCCACCCCTGTCGATAGGGAACGGTAGGGAACGGTAGGAACGGTGCTTTTC

GTGAACTGCAG
```

- 2. The gene fragment of claim 1 wherein said fragment is substantially free of extraneous DNA.
- 3. The gene fragment of claim 1 where the DNA is plasmid DNA.
- 4. The gene fragment of claim 1 where the source of the DNA is bacteria of the genus <u>Flavobacterium</u>.
- 5. The gene fragment of claim 1 where the source of the DNA is bacteria of the genus <u>Pseudomonas</u>.

6. An expression vector for producing bacterial organophosphorous acid anhydrase, said vector comprising a cloned bacterial organophosphorus acid anhydrase gene fragment having the DNA coding sequence:

```
CTSCAGCCTSACTCSGCACCAGTCSCTSCAAGCAGTCSTAAGCAATCSCAAGGGGGCACG
ATG CAA ACG AGA AGG STT STG STG AAG TCT SCS SCC SCA SGA ACT CTS CTC SGC
Sac gin the arg arg wal wal law lys see ala ala ala gly the law law gly
GGC CTG GCT GGG TGC GCG ACG TGG CTG GAT CGA TGA TGA CAG GGG ATC GGA TGA
gly leu ala gly cys ala the top leu asp any ser ala gin ala ile gly ser
 ATA COT GCG CCT CCT ATC ACA ATC TCT GAA GCG GCT TTC ACA CTG ACT CAC GAG ile arg ais arg pro ile thr ile ser glu als gly pne thr leu thr his glu
GAC ATC TOC GGC AGC TOS GCA GGA TTC TTS GGT GGT TGG GCA GAG TTC TTC GGT asp ile Gys gly ser ser als gly pne leu arg als tTP pro giu pne pne gly
 AGC COSC ANA GCT CTA GCS CAA AAG GCT GTS AGA GGA TTS CGC GCC AGA GCS GCT SER ERG lys ale leu ale glu lys ale val erg gly leu erg ale erg ale ale
GGC GTG CGA ACG ATT GTC GAT GTG TCG ACT TTT GAT ATT GGT CGC GAC GTC AGT Gly val ary the ile val asp val ser the pne asp ile gly ary asp val ser
TTA TTG GCC GAG GTT TCG CGG GCT GCC GAC GTT CAT ATC GTG GCG GCC ACC GGC leu leu ala glu val ser arg ala ala asp val his ile val ala ala thr fly
THE TOO THE GAC COS COA CTT TOS AND COA THE AGG TAT STA GAG GAA CTC ACA leu try pae asy pro pro leu ser met ary leu ary tyr val glu glu leu thr
CAG THE THE CHE COT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GCG gin one leu arg glu ile gin tyr gly ile giu asp thr gly ile arg ala
GGC ATT ATC AAG GTC GGC ACC ACA GGC AAG GCG ACC CCC TTT CAG GAG TTA GTG gly lie lie lys wal ala the the gly lys ala the pro one gin glu leu wal
THA AAG GCS GCC GCC GCC GCC AGC TTS GCC ACC GCT STT GCS GTA ACC ACT CAC leu lys ala ala ala ary ala ser leu ala the gly val pro val the the the
ACS SCA SCA AGT CAG CGC SAT SGT SAG CSA SGC AGG CGS CGA TITT TIS AGT CGG
ANG OTT GAG OTT TOA OGG OTT TOT ATT GOT CAC AGO GAT GAT ACT GAG GAT TTG
Lys law glu pro ser arg val dys lle gly his ser asp asp thr asp asp law
AGO TAT OTO AGO GOD GTS GTS GGC GGA TAG TTO ATO GGT GTA GAG GAG ATO GGS ser tyr leu thr sie leu leu erg gly tyr leu ile gly leu esp his ile pro
can not see all set out sal dat ant see her sea tea cos out the sec his ser all the gly leu glu asp ash all ser all ser pro leu leu gly the \frac{1}{2}
can now new call act one can one the the law does one and the call doe the any ser the gin the any all lew lew lie lys all lew ile asp gin gly tyr
ATS ANA CAN ATC CTC GTT TOS ANT SAC TSS CTS TTC GGG TTT TCS AGC TAT GTC net lys gin the leu val ser asn asp trp leu pne gly gne ser ser tyr val
אכל אאל אדל אדם כאל כדם אדם כאד ככל כדם אאל ככל כאל ככל אדם ככל דדל אדד
thr asmile met aspival met aspiarq valiasmipro aspigly met ala phe ile
cen ett han ett htte een tie the dag han has des tee ene his heg han ege tee
pro leu arg val ile pro pae tyr glu arg arg ala ser his heg lys arg cys
```

- 7. The expression vector of claim 6 further comprising a promoter, a start codon, and a recombinant DNA sequence coding for bacterial organophosphorus acid anhydrase in accurate reading frame sequence with said start codon for translation.
- 8. The expression vector of claim 7 wherein said vector is derived from a baculovirus.
- 9. The expression vector of claim 7 wherein said vector is a bacteriophage.
- 10. The expression vector of claim 7 wherein said vector is a plasmid.
- 11. The expression vector of claim 10 wherein said plasmid comprises a transposon capable of transposing the drosophila genome.

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12. A transformed microorganism comprising an expression vector for producing bacterial organophosphorus acid anhydrase wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment with the DNA coding sequence:

```
5'
 CTSCAGCCTGACTCGGCACCAGTCGCTGCAAGCAGAGCATCGTAAGCAATCGCAAGGGGGGCAGC
ATS CAA ACG AGA AGG GTT GTG TTC AAG TCT GCG GCC GCA GGA ACT CTG GTC GGC
get gin the arg arg val val lew lys ser ala ala gly the lew lew gly
 GGC CTG GCT GGG TGC GCG ACG TGG CTG GAT CGA TGA GCA CAG GCG ATC GGA TCA
gly lau ala gly cys ala thr trp lau asp arg ser ila gin ala ila gly ser
 ATA CST SCS CST CCT ATC ACA ATC TCT CAA GCS GST TTC ACA CTS ACT CAC CAG ile arg ala arg pro ile thr ile ser glu ala gly pne thr leu thr his glu
 GAC ATC TOC GGC AGC TCG GGA GGA TTC TTG GGT GCT TGG CCA GAG TTC TTC GGT asp lie cys gly ser ser ala gly pne leu ary ala trp pro glu pne pne gly
 AGC COC AAA GCT CTA GCG CAA AAG GCT GTG AGA SGA TTS CGC GCC AGA GCG GCT
ser arg lys ala leu ala glu lys ala val arg gly leu arg ala arg ala ala
GGC STS CGA ACG ATT GTC GAT GTG TCG ACT TTC GAT ATC GGT CGC GAC STC AGT GIV val arg thr lie val asp val ser the pne asp ile gly arg asp val ser
TIA TIG GCC GAG GTT TGG GGG GGT GCC GAC GTT CAT ATC GTG GGG GGG ACC GGC Lau lau ala glu val ser arg ala ala asp val his ile val ala ala thr gly
TIS ISS ITS SAC COS COA CIT TOS AIG COA TIS AGG TAT STA GAG GAA CITS ACA leu try pne amp pro pro leu mer met ary leu ary tyr val glu glu leu thr
CAG THE THE CHE COT GAG ATT CAA TAT SEC ATE SAA SAC ACE SEA ATT AGG SES
gin one one leu ary giu ile gin tyr giy ile giu asp thr gly ile ary sia
GOT ATT ATC AND GTC GCC ACC ACA GGC AND GCC ACC CCC TTT CAG GAG TTA GTC Gly lie lie lys val als the the gly lys als the pro one gin giu leu val
THA AAG GCG GCC GCC GCC AGC TTG GCC ACC GCT GTT TCG GTA ACC ACT CAC law lys ala ala ara ala ser law ala thr gly val pro val thr thr his
ACC SCA SCA ACT CAG COC SAT SCT SAG COA SCC ACG CCA TITT TOS ACT CCC
TAT BLE BLE SET GLE BET BED GLY GLE BET GLY BET DEO DEO Lee Set DEO
AAG OTT GAG COD TOA COG GTT TST ATT SOT DAG AGG SAT GAT AGT SAG SAT TTO
Lys lau glu pro ser arg val dys bla gly bis ser asp asp the asp asp lau
AGO TAT OTO AGO GOO GTS GTS GGC SGA TAG FTC ATC SGT GTA GAG CAG ATC SGS ser tyr lew thr six lew lew arg gly tyr lew tie gly lew asp his tie pro
כפה דכם דבם כאא אכא ככם ככד כדב דדם אדם אאם ככם כדב אדם כאם כאא ככם דאם
ary ser trp gln thr arg ala leu leu ile lys ala leu ile asp gln gly tyr
ATG AAA CAA ATC GTG GTT TGG AAT GAC TGG GTG TTG GGG TTT TGG AGG TAT GTG get lys gin lie leu val ser asn asp try leu pne gly pne ser ser tyr val
ACC AAC ATC ATG GAC GTG ATG GAT CGC GTG AAC CCC GAC GGG ATG GCC TTC ATT
this asmalle met asp valuet asp argival asm pro asp gly met ala pre ile
CCA CTS AGA GTG ATC CCA TTC TAC GAG AGA AGG GGG TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg gys
CAG GCA TCA CTG TGA
gin ala ser leu .
CTAACCCGGCGCGGTTCTG
ACTATTCCCCGTCAAGATA
```

- 13. The transformed microorganism of claim 12 wherein said microorganism is a bacteria.
- 14. A transformed eukaryotic cell line comprising an expression vector for producing bacterial organophosphorus acid anhydrase wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment with the DNA coding sequence:

```
GOC CTS GCT GGG TGC GCS ACS TGG CTS GAT CSA TGS GCA CAG GCS ATC SGA TCA
gly leu ala gly cys ala the top leu asp and ser sia gin ala lie gly ser
ATA COT OCC COT CCT ATC ACA ATC TOT GAA GCS GGT TTO ACA CTS ACT CAC GAG
tile arg ala arg pro tile thr tile ser giu ala gly one thr leu thr his giu
CAC ATC TOC CGC AGC TOS GCA GGA TTC TTG CGT GCT TGG CCA GAG TTC TTC GGT asp lie cys giv ser ser als gly pne leu arg als trp pro giu pne pne gly
 AGC COC AAA GCT CTA GCS CAA AAG CCT CTC AGA SGA TTS CCC SCC AGA SCS GCT ser arg lys ala leu ala glu lys ala val arg gly leu arg ala arg ala ala
 GGC STS CSA ACS ATT GTC GAT GTG TCS ACT TTC GAT ATC SGT CSC GAC GTC AGT GTY val arg the tile val asp val ser the pne asp tile gty arg asp val ser
 TTA TTS SEC SAG STE TOS SSG SCT SEC SAC STE CAT ATC STS SES SES ACC SSC leu leu ala glu val ser arg ala ala asp val his ile val ala ala che giy
  THE THE THE GAC COS COA COT TOG ATS COA THE AGE TAT STA GAG GAA CTO ACA leu try one amp pro pro leu ser met ary leu ary tyr wal glu glu leu thr
 the the the one can take aft that the set and that dad ago the gly lie and all
 GGC ART ARC AND GTC GGS ACC ACA GGC AND GGG ACC GGG TTT GAG GAG TTA GTG Gly tile tile lys wal ala gar gar gly lys ala gar pro pne gin giu leu wal
  TTA AND GOS GOS GOS GOS GOS AGO TTS GOS ACC GOT STT GOS STA ACC ACT CAC leu lys ala aia aia arg aia ser leu aia thr gly val pro val thr thr his
  yes dey yet yet eye ede and and and eny and the and bid big but the yes eed bid
  AND OTT DAG COO TOX COO OTT TOT ATT OUT CAG AGG DAT GAT ACT DAG DAT TTO
Lys law glu pro ser arg wal dys lie gly bis ser asp asp bur asp asp law
  AGO THE OTO AGO GOO GTO GTO GGG GGA THE GTO ATO GGT GTA GAC CAC ATO GGG Ser Tyr leu the siz leu leu ary gly tyr leu tie gly leu asp ats tie pro
    CAC ACT OCS ATT OCT CTA CAA CAT AAT OCS ACT OCA TCA COS CTC CTG GGC ATC
his ser ale ile gly leu glu esp esn ele ser ale ser pro leu leu gly ile
    con too too cay yey coo den one the the the ste one one cay doe cay coo cay and the table to the see the cay coo cay one table the ten the table table
    ATS ANA CAN ATC OTC STT TOS ANT SAC TOS OTS THE SGG THE TOS AGC THE STC DEC LYS GET LIE leu val ser asn asp top leu pae gly one ser ser typ val
    אכם אאם אדם אדם שאם סדם אדם מאד ככם סדם אאם ככם מאם שכם אדם שכם דדם אדד
    the asn lie met asp val met asp and val asn pro asp gly met als poe lie
    CCA CTS AGA GTS ATC CCA TTC TAC SAG AGA AGG GCS TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg cys
     CAB SEA TEA CTS TOA

91A 81A 88F 18U T

CTAACCESGESGETTETOTOTECACESACTISCOSTICATIACSCEATETIGATECTTICACSCAGESSEE

ACTATISCOSTICASSATACCESACESATGAAGTACCEATCGATAGGEATCTTCAATGTTATICACGG

ACTATISCOSTICASSATACCESACCECTISTICAATAGGEACCGTAGCGACCGTGCTTCACGG

CTSCCACCTICAAAGCCGGGTGGCCACCCCTTTCGATAGGTTCGACGACCGTAGCGACCGTGCTTTCG
     כעם פכא דכא כדים דכא
      متستتنده
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-53-

- 15. The transformed cell line of claim 14 wherein said cell line is derived from an insect.
- The transformed cell line of claim 15 wherein said insect is a Fall army worm caterpillar.
 - odd ott out odd tod odd Add tod ott oAT toA tod och ATC odA toA gly leu ala gly oys ala thr trp leu asp ary ser 11a gln ala ile gly ser ATA OST OSS OST OST ATO AGA ATO TOT SAA GGS GGT TTO AGA GTS AGT SAG SAG lie arg ala arg pro lie the lie ser glu ala gly pne the leu the his glu SAC ATC THE GOD AGE TOO GOA HOW THE THE COT BOT TOO COA GAS THE THE BOT AND LIE MYS GLY SEE SEE SIA GLY DOE LOU AND ALL TOP DET GLU DOE DOE GLY AGO COC AAA GCT CTA GCC GAA AAG GCT GTG AGA GGA TTG CGC GCC AGA GCG GCT ser arg lys ala leu ala glu lys ala val arg gly leu arg ala arg ala ala SGC STS CSA ACS ATT STC SAT STS TCS ACT TTS SAT ATC SST SSC SAC STC AGT gly val arg the val asp val ser the one asp the gly arg asp val ser THA THE ONE GAG STE TOO COS ONE SEE CAC STE CAT ATC STO GOS GOS ACC SEC Leu leu ala glu val ser are ala ala asp val his ile val ala ala the gry THE THE THE SAC COS COA CTT TOS ATS COA THE AGG TAT STA GAG GAA CTC AGA Leu try one amp pro pro leu mer net ary leu any tyr val glu glu leu the CAG THE THE STS OUT SAG ATT CAA TAT SSC ATT SAA SAC ACT SGA ATT AGG SGS gin one one led any gid tie gin byt gly tie gid asp the gly tie any ala SEC ATT ATC AND STC SCS ACC ACA SEC AND SES ACC CCC TIT CAS SAG TIX STG gly lie lie lys wal ale the the gly lys ale the pro one gin glu leu wal THE AND SES SES SES SES SES SES AGE THE SES ACC SET STT SES STA ACC ACT CAG Law Lys ala ala ala arg ala ser law ala the gly wal pro wal the the his ACC SCA SCA ACT CAG COC SAT SOT SAG SSA SOC AGG SES SCA TIT TIG ACT COS the six aix ser gin arg asp gly gin arg gly arg pro pro pre leu ser pro AND OTT THE COT TON COS OTT TOT ATT SOT CAC AGO SAT SAT ACT THE LYS LAW gir pro ser any war dys lie gir his sen asp and the asp asp law AGO TAT OTO AGO GOO OTS OTS OSG SGA TAG OTO ATO SGT OTA SAC CAC ATO SGS ser typ leu tar ale leu leu ary gly typ leu ile gly leu asp als ile pro the Act see ATT set tha sad sat ant see Act sea tha see off the see all see al con too too caa aca cod con one one and aad doo one and caa caa doc tac and ser top gin top and sie lee lee lie lys ale lee lie asp gin gly dyn ATS ANA DAN ATC CTC STT TOS ANT SAC TOS CTS TTC SGG TTT TOS AGC TAT GTC sec lys gin lie lew wal ser asn asp try lew pne gly pne ser ser tyr wal ACC AME ATO ATO DAG STO ATO DAT COD STO AME COD DAG DOG ATO DOC THE ATT the asn lie met asp val met asp arg val asn pro asp gly met ala pne lie COA OTO AGA GTO ATC COA TTC TAC DAG AGA AGG DCG TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg cys CAG SCA TCA CTS TSA

 gin sia ser leu

 CTAACCESSCESCATTOCSTSTCACCEACTTSCCSTSCATSACSCCATCTSGATCCTTSCACSCAGESGCE

 ACTATTSSCCSTCAAAGATACCGAACGATGAAGTCSGCATCSATGATAGGCATCTTCAATGTSTCAGGG

 CTGCCACCTCCAAAGCGSGTSGCCACCCTTSTCSATAGGGACGGTAGGTAGGACGACGGTGGTTTG

17. A transgenic eukaryotic organism comprising an expression vector for producing bacterial organophosphorus acid anhydrase wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment having the DNA coding sequence:

```
TISCHGGGTSACTGSGCACCAGTGSGTSCAGGAGGTGSTAAGGAATGSCAAGGGGGGCAGG
ATS CAA ACS AGA AGG STT STS STS AAG TOT SCS SCC SCA SGA ACT STS STS SGC
Set gin the arg arg wal wal leu lys ser sla ala ala gly the leu leu gly
see one sen see see see and the try led and and sex and des and sex fex gly led ala giv dyn ala the try led and any ser tha gin ala the gry ser
ATA COT GOS COT COT ATO ACA ATO TOT GAA GOS GOT THE ACA CTG ACT CAC GAG
the arg ala arg pro the the the ser giv ala giv one the lew the mas giv
she had too see ago too seh sea at the tre set set too can she tre tre set asp the cys giv ser ser ala giv pae leu arg ala tre pro giu eas ene giv
age one and get oth des sha had set sts aga sea tos see see aga tes set are lys ala leu ala glu lys ala val are gly leu are ala are ala ala
odd oto coa aco art otd dat otd too act the dat are due dad otd agr gly val ary the ile val asp val ser the one asp ile gly ary asp val ser
THA THE GOO GAG STT TOS OSS SOT SOC SAC STT CAT ATC STS SOS SOC ACC SOC leu leu ala glu val ser arg ala ala asp val his ile val ala ala thr gly
THE THE THE SAC SES CEA CTT TOS ATS COA THE AGS TAT STA GAG SAA CTT ACA leu try one amp ore ore leu mer met ary leu arg tyr wal giu jeu ten
cad the the ere can sad art caa hat see are sad sad acc sea art acc ses gin one one led art gid the gin byt giv the gid asp the giv the art als
ode ATT ATC AAG OTC OCC ACC ACA OGC AAG OCC ACC CCC TTT CAG GAG TTA STO giv lie lie lys wal als the the gly lys als the pro one gin giv leu wal
THE AAG SES SES SES SES SES SES AGE THE SES AGE SET STT SES STA AGE ACT CAG
law live alle alle and alle ser law alle the gly val pro val the the his
ACC SCA SCA ACT TAG COC SAT SOT SAG SCA SCC ACC SCS SCA TIT TIS ACT COS TAT all ala ser gin arg asp gly glu arg gly arg pro pro pae leu ser pro
AND OTT DAG COT TOA COG OTT TOT ATT OUT TAC AGO DAT DAT ACT DAG DAT TOO
Lys law glu pro ser arg val dys lie gly his ser asp aso the asp asp law
AGO TAT THE AGO GOD OTS CTS CGC SGA TAC THE ATO SGT THA SAC CAC ATO TOS SET TYP lew the six lew lew ary gly typ lew tie gly lew asp cas tie pro-
THE ACT SES ATT SET CTA SHA GAT ART SES ACT SEA TEA CES STE STE SEE ATE THE SET ALL SE
com red red cal Aca ess sem eme the Ate and des eme and cal cal sec the are sen tro gla the ang all leu leu lle lys all leu ile amp gla gly typ
ATG ANA CAN ATC CTC STT TCG ANT SAC TGG CTC TTC SGG TTT TCG AGC TAT GTC cec lys gin ile leu val ser asn asp trp leu pne gly pne ser ser tyr val
ACC AAC ATC ATG SAC STG ATG SAT CSC STS AAC CCC SAC SGS ATG SCC TTG ATT
the asn the met asp valuet asp are values pro asp gly met als one the
COA STO AGA OTG ATC COA TTO TAC GAG AGA AGG GGG TGC CAC AGG AAA CGC TGC pro leu arg vai ile pro pne tyr glu arg arg ala ser his arg lys arg cys
CAG SCA TCA CTS TGA

917 814 88F 18U

CTAACCESGCSCSGTTCTCSTSTCACCSACTTSCCSTTGCATSACGCCATCTSGATGCTTTCCACGCAGGGGGGC

ACTATICSCSSTTANGATACCSAACGATGAGGTTCCACTGATGCATTCCACGCAGGGGGGC

ACTATICSCSSTTANGATACCGAACGATGAAGTTCGATAGGGAACGGTAGGGACGGTGCTTCAATGTATCAGGG

CTSCCACCTGCAAAGCCGGTGGCCACCCCTTSTCGATAGTTTTTAAGGGAACGGTAGGGACGGTGCTTTTTT

GTGAACTGCAG

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18. A transgenic organism as claimed in claim 17 wherein said organism is derived from microinjection of said expression vector into drusophila melanogaster embryo cells.

csm

- 19. A transgenic organism as claimed in claim 17 wherein said organism is derived from injection of said expression vector into a Fall army worm caterpillar.
- 20. A method for making bacterial organophosphorus acid anhydrase, said method comprising:
 - growing in a nutrient medium a transformed microorganism having an expression vector with a cloned bacterial organophosphorus acid anhydrase gene fragment having the DNA coding sequence:

TISCAGECTISACTESSECACEAGTESCTCELAGEAGATCSTRAGEAATCSCRAGGGGGGCAGC
ATS CAA ACG ACG ACG STT STG STC AAG TOT SCG SCC SCA SGA ACT STS STC SGC
SEC SIR INT SET SET WELL VALUE Lys ser sia ata ala gly the leu leu gly SEC CTS SET SEC SEC SES ACS TSE CTS SAT CEA TOS SEA CAG SES ATC SEA TEX Fly leu ala gly cys ala the trp leu asp ary ser lia gln ala lle gly ser ATA COT GCC COT CCT ATC ACA ATC TOT TAA GCC COT TTC ACA CTC ACT CAC CAG lie arg ala arg pro lie thr lie ser glu ala gly pne thr leu thr his glu GAC ATC TOC GGC AGC TOS GGA SGA TTC TTS GGT GGT TGG GGA GAG TTC TTC SGT asp lie cys gly ser ser ala gly pne lau arg ala trp pro glu pne pne gly AGC ISC AAA GCT OTA GCG GAA AAG GCT STS AGA GGA TTS ISC ISC AGA ISC ISCT INT LYS ala leu ala glu lys ala val arg gly leu arg ala arg ala ala SSC STS CGA ACS ATT GTC GAT GTG TCS ACT TTC GAT ATC SST CSC GAC STC AGT GLY val ary the lie val asp val ser the pne asp lie gly ary asp val ser THA THE GCC GAG STT TOS COS SCT SCC CAC STT CAT ATC STS CCS GCC ACC SCC leu leu ala giu val ser arg ala ala asp val his ile val ala ala chr gly THE TOO THE GAC CES COA CTT TOO ATS COA THE AGO TAT STA GAG SAA CTC ACA CAG THE THE CHE COT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GCG glm pne pne leu arg glu ile gin tyr gly ile glu asp thr gly ile arg ala GGC ATT ATC ANG STC GGS ACC ACA GGC ANG GGS ACC GGC TTT CAG GAG TTA GTG gly the the lys wal als the the gly lys als the pro one gin glu leu wal TTA AAG GOG GOG GOG GOG GOG AGG TTS GOG AGG GOT STT GOG GTA AGG AGT GAG leu lys ala ala ala arg ala ser leu ala thr gly val pro val thr thr his ACC SCA SCA ACT CAG CCC SAT GCT SAG CCA SCC ACG CCS CCA TTT TTO ACT CCC CAT all all set gin arg asp gly glu arg gly arg pro pro pue leu set pro AND CTT DAG COO TON COO GTT TOT ATT OUT CAC AGE OUT OUT ACT DAG OUT TOO Lys law glu pro ser arg val dys lie gly his ser asp asp thir asp asp law AGO TAT OTO ACO GOD OTS OTS OGG SGA TAC OTO ATO GOT OTA GAC CAC ATO GOS SET TYP leu the sia leu leu arg gly typ leu tie gly leu asp his tie pro CAC AGT SGS ATT SGT CTA SAA GAT AAT SGS AGT SGA TGA GGS GTG GTG GGG ATG HIS ser ala ile gly leu glu asp asn ala ser ala ser pro leu leu gly ile out for the dal aca one get one the are and see one are tak one take any ser try gin the ary all leu leu lie lys all leu ile asy gin gly tyr ATS AAA CAA ATC CTC GTT TOS AAT SAC TGG CTG TTC GGG TTT TGG AGC TAT GTC net lys gln ile leu val ser asn asp trp leu pne gly pne ser ser tyr val אכם אאם אדם באם פדם אדם פאד ככם כדם אאם ככם באם ככם אדם ככם דדם אדד thr asm ile set asp val met asp arg val asm pro asp gly met ala pne ile CCA CTG AGA GTG ATC CCA TTC TAC GAG AGA AGG GCG TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg cys

allowing said microorganism to produce bacterial organophosphorus acid anhydrase; and

recovering the bacterial organophosphorus acid anhydrase.

21. A method for making bacterial organophosphorus acid anhydrase, said method comprising:

growing in a nutrient medium a transformed eukaryotic cell line comprising an expression vector with a cloned bacterial organophosphorus acid anhydrase gene fragment having the DNA coding sequence:

CTSCAGCCTSACTCSSCACCAGTCSCTSCAAGCAGAGCTCSTAAGCAATCSCAAGGGGGCAGC ATS CAA ACG AGA AGG STT STG TTS AAG TCT SCS SCC SCA SGA ACT STG STG SGC Set gin the arg arg val val leu lys ser sia ala giy the lau lau giy GGC CTG GCT GGG TGC GCG ACG TGG CTG SAT TGA TGG GCA AAG GCG ATC GGA TGA gly leu ala gly Gys ala the top leu asp arg sar ala gin ala ile gly sar ATA COT COT COT ATC ACA ATC TOT GAA GCG COT TOT ACA CTC ACT CAC CAG LIe ary ala ary pro lie the lie ser glu ala gly pne the lau the his glu SAC ATC TSC SGC AGC TGS SGA SGA TTC TTS CST SGT TGS GGA GAG TTC TGC SGT asp the cys gly ser ser ala gly pne leu arg ala tmp pro glu pne one gly AGC CSC AAA GCT CTA GCS SAA' AAG SCT GTS AGA SGA TTS CSC SCC AGA SGS GCT ser sry lys ala leu ala glu lys ala val ary gly leu ary ala ary ala ala SGC STS CGA ACS ATT STC GAT STG TCS ACT TTC GAT ATC GGT CGC GAC STC AGT gly val arg thr lie val asp val ser the one asp lie gly arg asp val ser TTA TTG GCC GAG GTT TGG GGG GGT GCC GAC GTT GAT ATG GTG GGG GGG AGG GGG lau lau ala glu val ser arg ala ala asp val his ile val ala ala thr gly TTO TOO TTO GAC COS CCA CTT TOO ATO COA TTO AGO TAT STA GAG SAA CTO ACA leu try one aso pro pro leu ser dec ary leu ary tyr wal glu glu leu thr cad the the end cot dad art can hat doe are dad dae ace doa art add des gin one one leu arg giu ile gin tyt gly ile giu asp the gly ile arg dia see Are Are had one see Ace Ach and see Ace see fire the gha the gray lie lie lys was als the the gly lys als the pro one gin giv les was THA AAG GOD GOD GOD GOD GOD AGO TTG GOD AGO GOT STT GOG GTA AGO AGT GAG leu lys ala ala ala arg ala ser leu ala the gly val pto val the the bis ACC SCA SCA ACT CAG CGC SAT SGT SAG CSA SGC AGG CGS CGA TTT TTG AGT CGS CAF alla ser gim arg asp gly glu arg gly arg pro pro pae leu ser pro AND OTT SAG COS TON COS OTT TOT ATT SOT CAC AGO SAT SAT ACT SAC SAT TTS Lys law glu pro ser arg val dys tie gly his ser isp asp the isp asp law AGO TAT OTO AGO GOO GTO GTO GGG GGA TAG GTO ATO GGT GTA GAG GAG ATO GGG ser typ leu chr ale leu leu arg gly typ leu lle gly leu asp glo lle gro the act off art off the sha sat hat off act for the tre tre off are his ser als the gly led giv asp ash als ser als ser pro led led gly the out too too dad aca coo dot oto the ate aad dos oto ate aac dad dod tac ang ser trp gin the arg ala leu leu lie lys ala leu lie asp gin gly tyt ATO ANA CAN ATC OTO STT TOO ANT SAC TSG OTO TTO SGG TTT TOO AGG TAT STC Dec lys gin lie leu wal ser ash asp trp leu pne gly pne ser ser tyr wal ACC AME ATC ATG GMC GTG ATG GMT GGC GTG AMC GGC GMC GGG ATG GGC TTC ATT the asn ile met asp val met asp arg val asn pro asp gly met ala pne ile can come and attended the take take and age one the take and the proper law and wall lie proper typ glu and and all sen his and lys and typ.

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allowing said microorganism to produce bacterial organophosphorus acid anhydrase; and

recovering the bacterial organophosphorus acid anhydrase.

- 22. A method for making bacterial organophosphorus acid anhydrase, said method comprising:
 - nourishing a transformed host in a nutrient medium allowing said host to produce bacterial organophosphorus acid anhydrase;
 - transforming host an expression vector comprising a DNA sequence coding for said bacterial organophosphorus acid anhydrase; and
 - separating the bacterial organophosphorus acid anhydrase from said host and said nutrient medium.
- 23. The method for making bacterial organophosphorus acid anhydrase of claim 22 further comprising purifying said bacterial organophosphorus acid anhydrase.
- 24. The method for making bacterial organophosphorus acid anhydrase of claim 22 wherein said host is a microorganism.
- 25. The method for making bacterial organophosphorus acid anhydrase of claim 24 wherein said microorganism is a bacteria.
- 26. The method for making bacterial organophosphorus acid anhydrase of claim 22 wherein said host is a eukaryotic cell line.

27. The method for making bacterial organophosphorus acid anhydrase of claim 26 wherein said eukaryotic cell line is derived from an insect.

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28. The method for making bacterial organophosphorus acid anhydrase of claim 27 wherein said insect is a Fall army worm caterpillar.

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29. The method of claim 22 wherein said anhydrase is purified to a level of approximately 3200 units/mg of anhydrase.

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30. The cloned bacterial organophosphorus acid anhydrase gene fragment of claim 1 where in the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

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31. The expression vector of claim 6 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

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32. The transformed microorganism of claim 12 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

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33. The transformed eukaryotic cell line of claim 14 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

34. The transgenic eukaryotic organism of claim 17 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

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35. The method for making bacterial organophosphorus acid

35. The method for making bacterial organophosphorus acid anhydrase of claim 20 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

- 36. The method for making bacterial organophosphorus acid anhydrase of claim 21 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.
- 37. The cloned bacterial organophosphorus acid anhydrase gene fragment of claim 1 wherein the C-terminal sequence
 20 has been deleted from Bam HI to PstI of said DNA coding sequence.
- 38. The expression vector of claim 6 wherein the C
 25 terminal sequence has been deleted from Bam HI to PstI of said DNA coding sequence.
- 39. The transformed microorganism of claim 12 wherein the 30 C-terminal sequence has been deleted from <u>Bam</u> HI to <u>Pst</u>I of said DNA coding sequence.

40. The transformed eukaryotic cell line of claim 14 wherein the C-terminal sequence has been deleted from Bam HI to PstI of said DNA coding sequence.

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41. The transgenic eukaryotic organism of claim 17 wherein the C-terminal sequence has been deleted from $\underline{\mathsf{Bam}}$ HI to $\underline{\mathsf{PstI}}$ of said DNA coding sequence.

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42. The method for making bacterial organophosphorus acid anhydrase of claim 20 wherein the C-terminal sequence has been deleted from <u>Bam</u> HI to <u>Pst</u>I of said DNA coding sequence.

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- 43. The method for making bacterial organophosphorus acid anhydrase of claim 21 wherein the C-terminal sequence has been deleted from Bam HI to PstI of said DNA coding 20 sequence.
- 44. Organophosphorus acid anhydrase produced by a genetically transformed host having an expression vectorcomprising a DNA sequence coding for said anhydrase.
 - 45. The method of claim 44 wherein said anhydrase is characterized by $K_{cat} = 2100 \text{ sec}^{-1}$ for paraoxon.

46. Bacterial organophosphorus acid anhydrase produced by a genetically transformed host having an expression vector comprising a cloned gene fragment with the DNA coding sequence:

```
GGC CTG GCT GGG TGC GCG ACG TGG CTG GAT CGA TGA CAG GCG ATC GGA TGA
gly leu ala gly cys ala the trp leu asp arg ser ala gin ala ile gly ser
 ATA COT GCG COT COT ATC ACA ATC TOT GAA GCG GGT TTC ACA CTG ACT CAC GAG ile arg ala arg pro ile the ile ser giu ala gly one the lau the his giu
GAC ATC THE GGC AGC TES HEA GGA TTO THE COT HET THE GGT AND THE THE HEA AND LIE GYS GLY SET SET ALE GLY DOE LEW AND ALE THE PRO GLY DOE PINE GLY
 AGC COC ANN GCT CTN GCD CAN ANG GCT GTD AGN GGA TTD CGC GCC AGN GCD GCT ser ary lys ale leu ale glu lys ale val ary gly leu ary ale ary ale ale
GGC STG CGA ACG ATT GTC GAT GTG TCG ACT TTC GAT ATC GGT CGC GAC STC AGT GLY val arg thr ile val asp val ser the one asp ile gly arg asp val ser
TIA TIG GCC GAG STT TGG GGG GCT GCC GAC STT GAT ATC STG GCG GCC ACC GGC leu leu ala glu val ser arg ala ala asp val his ile val ala ala thr gly
TTS TOS TTO GAC COS COA CTT TOS ATS COA TTO AGS TAT STA GAG GAA CTC ACA leu try pne asp pro pro leu ser nec ary leu ary tyr val glu glu leu tnr
CAG TTC TTC CTG CGT GAG ATT CAA TAT GGC ATC SAA GAC ACC GGA ATT AGG GCG
gin one one leu arg glu ile gin tyr gly ile giu asp tir gly ile arg ala
GGC ATT ATC AND GTC GGS ACC ACA GGC ANG GGS ACC GGC TTT CAG GAG TTA GTG
gly lie lie lys wal als the the gly lys als the pro gae gla glu leu wal
THA AND GOD GOD GOD GOD GOD AGO THE GOD AGO GOT GIT GOD GIA AGO AGO CAN law lys ala ala ala ary ala ser law ala the gly val pro val the the mis
ACS SCA SCA AGT CAG CSC SAT SGT SAG CSA SGC AGG CCS CCA TIT TITS AGT CCS
The six aix ser gin arg asp gly glu arg gly arg pro pro pre leu ser pro
AND CTT GAG CCC TCA CCC CTT TGT ACT GCT CAC AGC GAT GAT ACT GAC GAT TTG
Lys lau glu pro ser arg val cys lle gly his ser asp asp thr asp asp lau
AGO TAT OTO AGO GGO GTS GTS GGG GGA TAG GTC ATC GGT GTA GAG CAG ATC GGS ser tyr leu thr ale leu leu arg gly tyr leu ile gly leu asp his ile pro
CAC AGT GGS ATT GGT GTA GAA GAT AAT GGS AGT GGA TGA GGS GTG GTG GGC ATG his ser ale ile gly leu glu esp esn ele ser ale ser pro leu leu gly ile
COT TOO TOO CAA ACA COO GCT CTC TTC ATC AAG CCC CTC ATC CAC CAA GGC TAC
ary ser try gin the ary ala leu leu ile lys ala leu ile asp gin gly tyr
ATO AAA CAA ATC OTO GTT TOO AAT OAC TOO OTO TTO GGG TTT TOO AGO TAT GTC net lys glm ile leu val ser asn asp trp leu pne gly pne ser ser tyr val
ACC AAC ATC ATG GAC GTG ATG GAT CGC GTG AAC CCC GAC GGG ATG GCC TTC ATT
thr ash ile set asp val met asp arg val ash pro asp gly met als pne ile
CCA CTS AGA GTS ATC CCA TTC TAC GAG AGA AGG GCS TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg cys
כאם ככא דכא כדם דכא
CTSCERCETCEARINGCESGTGGCCACCCTTGTCGATAGTCTTGAGGGACGGTAGCGACGACGGTGC
GTSAACTSCAG
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47. The bacterial organophosphorus acid anhydrase of claim 46 wherein the N-terminal sequence up to the start codon has been deleted from said DNA coding sequence.

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48. The bacterial organophosphorus acid anhydrase of claim 46 wherein the C-terminal sequence up to the start codon; has been deleted from <u>Bam</u> HI to <u>PstI</u> of said DNA coding sequence.

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49. The bacterial organophosphorus acid anhydrase of claim 46 wherein said host producing acid anhydrase is a microorganism.

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50. The bacterial organophosphorus acid anhydrase of claim 46 wherein said host producing said anhydrase is a bacteria?.

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51. The bacterial organophosphorus acid anhydrase of claim 46 wherein said host is a eukaryatic cell line.

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52. The bacterial organophosphorus acid anhydrase of claim 46 wherein said anhydrase is relatively pure, characterized by $K_{\text{cat}} = 2100 \text{ sec}^{-1}$ for paraoxon.

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53. A method for detoxifying an organophosphorus compound comprising exposing said compound to recombinant bacterial organophosphorus acid anhydrase.

54. The method of claim 53 wherein said exposure is accomplished by passing said compound through a matrix comprising said recombinant anhydrase.

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- 55. The method of claim 54 wherein said matrix is further comprised of a filtration device.
- 10 56. The method of claim 55 wherein said device is a gas mask.
- 57. The method of claim 53 wherein said organophosphorus 15 compound is in air.
 - 58. The method of claim 53 wherein said organophosphorus compound is in a fluid.

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59. The method of claim 53 wherein said exposure is accomplished by spraying said recombinant anhydrase on a locus comprising the organophosphorus compound.

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60. The method of claim 53 wherein said exposure is accomplished by introducing said anhydrase into a container comprising the organophosphorus compound.

61. The method of claim 53 wherein said recombinant bacterial organophosphorus acid anhydrase is produced by a transformed microorganism comprising an expression vector for producing said anhydrase and wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment with the DNA coding sequence:

CTICAGCCTSACTCSGCACEAGTCSCTSCAAGCAGTCSTAAGCAATCSCAAGGGGGGAGCACCATCS CAA ACG AGA AGG GTT GTG GTC AAG TCT GCG GCC SCA SGA ACT GTS GTC GGC AGA ACT GTS GTC GGC AGA ACT GTS GTC GGC AGA ACT GTS GTC AGG ACT GTS GTC AGG ACT GTS GTC AGA ACT GTS GTC AGA ACT GTS GTC AGG ACT GTS GTC AGA ACT GTS AGA ACT AGA AC GGC CTS GGT GGG TGC GGS ACG TGG CTS GAT CGA TGA GGS ACG GGG ATC GGA TGA GLY leu ala gly cys ala the trp leu asp arg ser ala gin ala ile gly ser ATA COT GCG CCT CCT ATC ACA ATC TOT GAA GCG GCT TTT ACA CTG ACT CAC GAG ile arg als arg pro ile thr ile ser giu als gly pne thr leu thr his giu GAC ATC THE GGC AGC TOS GGA GGA THE THE COT HOS CHA GAG THE THE GGT asp lie cys gly ser ser als gly pae leu arg als the pro glu pae pas gly AGC COC AAA GCT CTA GCG CAA AAG GCT GTG AGA GGA TTG CGC GCC AGA GCG GCT ser arg lys ala leu ala glu lys ala val arg gly leu arg ala arg ala ala GGC STS CGA ACG ATT GTC GAT GTG TCG ACT TTC GAT ATC GGT CGC GAC STC AGT gly val arg the ile val asp val ser the pne asp ile gly arg asp val ser THA TTO GOO GAG STT TOO COO GOT SOO GAC STT CAT ATC STO GOD GOD ACC SOC LOU also giv values are are also also values the value also the giv THE TOO THE GAC CES CEA CTT TOO ATS COA THE AGO TAT CTA GAG SAA CTC ACA low try one asp pro pro low ser met ary low ary tyr wal glu glu low thr CAG THE THE CHS COT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GGG gin phe phe leu arg giu ile gin tyr giy ile giu asp thr giy ile arg ala GGC ATT ATC AAG GTC GGG ACC ACA SGC AAG GGG ACC CCC TTT CAG GAG TTA GTG gly tie tie lys wal ale that the gly lys ale that pro one gln glu leu wal TTA AAG GCG GCC GCC GGG GCC AGC TTG GCC AGC GGT GTT CCG GTA ACC ACT CAC leu lys ala ala ala arg ala ser leu ala thr gly val pro val thr thr his ACS SCA SCA AGT CAG CGC GAT SGT SAG CGA SGC AGG CGS CGA TTT TIS AGT CGG TAT ala ala ser gin arg asp gly glu arg gly arg pro pro poe leu ser pro AND CTT GAG COD TOA COG GTT TOT ATT GOT CAC AGO GAT GAT ACT GAC GAT TTO Lys law gim gro ser arg val dys ble giv his ser asp asp thr asp asp law AGG TAT OTG AGG GGG GTS CTS GGG GGA TAG STG ATG GGT GTA GAG GAG AGG GGG ser gyr leu thr sia leu leu arg gly gyr leu ile gly leu asp his ile pro can agt one att out tha saa gat aat one agt sea tea one otto otto doo att his ser saa ile gly leu glu asp ash ela ser saa ser pro leu leu gly lle con now now can how one con one one had some one one had the can one the arg ser trop gin the arg all leu leu lie lys all leu lie asp gin gly tyr ATS ANA CAN ATC OTC STT TOS ANT SAC TGG CTS TTC GGG TTT TCS AGG TAT GTC det lys η in lie leu val ser asn asp trp leu pne η ly pne ser ser tyr val ACC AAC ATC ATG GAC GTG ATG GAT GGG GTG AAC GGG GAC GGG ATG GGC TTC ATT thr asn ile met asp val met asp arg val asn pro asp gly met ala pne ile CCA CTS AGA GTS ATC CCA TTC TAC GAG AGA AGG GCS TCC CAC AGG AAA CGC TGC pro leu arg val ile pro pne tyr glu arg arg ala ser his arg lys arg cys כגב בכא דכא כדה דכא

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The method of claim 53 wherein said recombinant bacterial organophosphorus acid anhydrase is produced by a transformed eukaryotic cell line comprising an expression 5 vector for producing said anhydrase and wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment with the DNA coding sequence:

> CTSCLAGGETSACTOSSCLAGGAGTGSCTAGGAGATGTTAAGGAATGSCLAGGGGGSGLAGG ATG CAA AGG AGA AGG GTT GTG GTC AAG TGT GCG GCC SCA SGA AGT GTS GTC SGC Sec gin the erg erg val val leu lys ser als als als gly the leu leu gly GOC CTS GCT GGG TGC GCS AGG TGG CTS GAT CAA TGS SCA CAG GCS ATC SGA TGA gly leu ala gly Gys ala the trp leu asp arg ser ala gin ala ile gly ser ATA COT GCG COT CCT ATC ACA ATC TCT GAA GCG GGT TTC ACA CTG ACT CAC GAG ile arg ais arg pro ile the ile ser glu ais gly pne the leu the his glu GAC ATC THE GGC AGC THE GEA GGA THE THE COT GCT THE CEA GAG THE THE GGT asp lie cys gly ser ser als gly pas leu arg als trp pro glu pas pas gly AGC COC AAA GCT CTA GCG GAA AAG GCT GTG AGA GGA TTG CGC GCC AGA GCG GCT ser ary lys ala leu ala glu lys ala val ary gly leu ary ala ary ala ala ode one can ace are die dat one not the sar are dot one ase gly val are the ile val ase val ser the one ase ile gly are ase val ser TTA TTS GCC GAG GTT TCG CGG GCT GCC GAC STT CAT ATC GTG GCG GCG ACC GGC leu leu ala glu val ser arg ala ala asp val his ile val ala ala chr gly THE THE THE GAC COS CCA CTT TOS ATS CEA THE AGE TAT GTA CAG GAA CTC ACA lew try pae asy pro pro lew ser nec ary lew are typ wal glw lew thr CAG THE THE CHE COT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GGS gin pne pne leu arg giu ile gin tyr giy ile giu asp thr giy ile arg ala GGC ATT ATC AND OTO GGS ACC ACA GGC AND GGS ACC CCC TTT CAN GAG TTA GTG gly the the typ wat ale the the gly typ ale the pro pne gin giv leu wat THE AND GOTS GOT SET COS GOT AGO THE GOT ACT GOT GIT COS GIR ACT ACT CAC leu lys ala ala ala arg ala ser leu ala ter gly val pro val the ter his ACC CON CON ACT CAG COC CAT GOT GAG COA COC AGG CCS CON TIT TTO ACT CCC THE SIA ala ser gin are asp gly glu are gly are pro pro pae leu ser pro AND CTT GMG CCC TON CGG GTT TOT ATT GGT CAC AGG GAT GAT ACT GAG GAT TTG Lys lau glu pro ser arg val dys ile gly his ser asp asp the asp asp lau AGE THE CTO ACC GCC CTS CTS CSC GGA THE CTO ATC SGT CTA GAC CHE ATC CCS ser Tyr leu the ale leu leu ary gly tyr leu ile gly leu asp his ile pro CAC AGT GGS ATT GGT CTA GAA GAT AAT GGS AGT GGA TGA GGS GTG GTG GGG ATG his ser als ile gly leu glu asp asn als ser als ser pro leu leu gly ile כפר דכם דבם כאג אכא ככם ככד כדכ דדם אדכ אגם ככם כדם אדכ באכ כאג ככב דאכ ary ser try gin the ary ale leu leu ile lys ale leu ile asp gin gly tyr ATG AAA CAA ATG GTG GTT TGG AAT GAG TGG GTG TTG GGG TTT TGG AGG TAT GTG get lys gln ile leu val ser asn asp trp leu pne gly pne ser ser tyr val ACC AME ATC ATG SAC GTG ATG GAT GGG GTG AME GGG GAC GGG ATG GGC TTG ATT thr asmile met asp val met asp arg val asm pro asp gly met ala pne ile cms and are are cen the the dag and and see fire car and and cen fre pro leu are val ile pro one tyr glu are are all ser his are lys are cys CLG GCA TCA CTG TGA
>
> gin ala ser leu
>
> CTARCESSGESCHTETETETETECCEARCTTGCGTGCATGACGCCATGTTGCATGCCTTCCACGCAGCGGCG
>
> ACTATTCCCCGTCTARGATACCGAAACGATGAGTGCGTGCATGCATGCATAGGCATCTTCAATGTATACAGGG
>
> CTGCCACGTCGAAAGCCGGTGGCCACCGCTGTCGATAGGGAACGGTAGGGAACGACGGTGCTTTCA

63. The method of claim 53 wherein said recombinant bacterial organophosphorus acid anhydrase is produced by a transgenic eukaryotic organism comprising an expression vector for producing said anhydrase wherein said vector has a cloned bacterial organophosphorus acid anhydrase gene fragment with the DNA coding sequence:

CTSCAGGGTSAGTCSGGCACCAGTCSGTSGAAGCAGTGSTAGGCAATGSCAAGGSGGGAGGATGS GAA AGG ATT STG STG AAG TOT SCG SCC SCA SGA ACT STG STG SGC SGA ACT STG STG SGC SGA ACT SGA ACT SGA ACT SGA ACT STG SGC SGA ACT SGA SEC CTS GCT GGG TGC GCS ACS TGG CTS GAT CGA TGA TGA CAG GCS ATC GGA TGA gly leu ala gly Gys ala the tup leu asp ary ser ila gin ala ile gly ser ATA COT GOS COT COT ATC ACA ATC TOT SAA GOS GOT TTO ACA CTO ACT TAC GAG lie ary sia arg pro lie the lie ser glu sia gly one the leu the his glu SAC ATC THE GOT AGE THE GEA GEA THE THE CHT COT THE CEA GAG THE THE SOT AND THE GOT AGE THE THE SOT age one and loss als less als glu lys als val and gly less and als als als als SSC STS CGA ACS ATT STC GAT STS TCS ACT TTS GAT ATS SST CSC GAC STC ACT gly wal arg the tile wal asp wal ser the one asp tile gly arg asp wal ser TTA TTG GCC GAG GTT TGG GGG GCT GCC GAC GTT CAT ATC GTG GCG GCG ACC GGC leu leu ala glu val ser sry ala ala asp val his ile val ala ala chr giy THE TOO THE GAC SES CEA CIT TOO ATO COA THE AGO TAT STA GAG GAA CITE ACA LOU TEP pine amp pine pro lou mor not any lou may typ wal glu glu lou the CAG THE THE CHS COT GAG ATT CAA TAT GGC ATC GAA GAC ACC GGA ATT AGG GGS gin one one leu ary glu ile gin tyr gly ile giu asp thr gly ile ary sia SEC ATT ATO AND STO SES ACC ACA SEC AND SES ACC CCC TITT CAS SAG TIA STE gly lie lie lys wal ala the the gly lys ala the pro pne-gin glu leu wal TTA AND GOD GOD GOD GOD GOD AGO TTO GOD NOT STT GOD GTA AGO ROT CAG law lys ala ala ala ary ala ser law ala the gly val pro val the the his ACC SCA SCA ACT CAG COC SAT SCT SAG COA SGC AGG COS CCA TIT TIS ACT COS TAT SIA ALA SET GIA AFG ASD GLY GLU AFG GLY AFG DED DED DED LEU SET DED AND CTT SAG CCC TOA COG GTT TOT ATT SOT CAC AGO SAT SAT ACT SAC SAT TTO Lys lau glu pro ser arg wal dys lie gly his ser asp asp the asp asp lau AGO TAT OTO AGO GGO GTS GTS GGO SGA TAG TTO ATO SGT GTA SAG GAG ATO GGS ser tyr leu thr ala leu leu arg gly tyr leu ile gly leu asp his ile pro CAC AGT GGS ATT GGT CTA SAA GAT AAT GGS AGT GGA TGA GGS GTG GTS GGG ATG his ser sla ile gly leu glu asp asn ala ser sla ser pro leu leu gly ile con now now can how con out one has and had one out and the day one the arm sen upp gin the arm all lew lew lie lys all lew lie asp gin gly typ ATS AAA CAA ATC OTC STT TOS AAT SAC TSS OTS TTC SGS TTT TOS AGC TAT STC Sec lys glm ile leu val ser asm asp trp leu pne gly pne ser ser tyr val ACC AAC ATC ATG GAC STG ATG GAT CGC STG AAC CCC GAC GGG ATG GCC TTC ATT the asm lie met asp val met asp arg val asm pro asp gly met ala pne lie cox cts AGA cts Ate cex the the she AGA AGG des tee che AGG AAA ede tee pro leu arg wal ile pro pne tyr glu arg arg ala ser his arg lys arg cys

64. A method of preventing poisoning of a locus by an organophosphorus compound by applying recombinant organophosphorus acid anhydrase to said locus before said compound contacts said locus.

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65. A method of detecting bacterial colonies capable of detoxifying organophosphorus acid anhydrides, comprising employing a transformed microorganism as a control in a plate assay wherein said microorganism is comprised of an expression vector for producing organophosphorus acid anhydrase and said vector is comprised of a cloned gene fragment containing the DNA coding sequence for the anhydrase.

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- 66. The method of claim 65 wherein said anhydrides comprise a pesticide and said plate assay is conducted prior to applying said pesticide to soil to quantitate the number of microorganisms capable of detoxifying said pesticides in said soil.
- 25 67. A method for protecting insects from organophosphorus compounds comprising feeding said insects recombinant organophosphorus acid anhydrase.
- 30 68. A method for protecting insects from organophosphorus compounds comprising infecting insects with microorganisms comprised of an expression vector for producing an organophosphorus acid anhydrase wherein said vector is comprised of a cloned gene fragment containing the DNA

35 coding sequence for the anhydrase.

- 69. A method for protecting insects from organophosphorus compounds comprising introducing into the environment of said insects microorganisms comprised of an expression vector for producing an organophosphorus acid anhydrase wherein said vector is comprised of a cloned gene fragment containing the DNA coding sequence of the anhydrase.
- 70. A pesticide comprising an organophosphorus compound10 and an inhibitor of bacterial organophosphorus acid anhydrase.

ABSTRACT

The bacterial gene (opd) encodes an organophosphorus anhydrase which is capable of hydrolyzing a wide spectrum of neurotoxins ranging from insecticides to mammalian neurotoxins. The cloned gene has been expressed in a number of hosts and the purified enzyme has been characterized. These advances have led to a number of interrelated uses for the cloned gene and the recombinant enzyme including: detoxification of organophosphorus compounds; protection of susceptible organisms from organophosphorus poisoning; and, detection of organophosphorus-detoxifying microorganisms.

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